이학박사학위논문

P19 배아줄기 암세포의 신경세포 분화과정에서 세포분열의 역할에 관한 연구

Studies on the role of cell division during neuronal differentiation in P19 EC cells

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Abstract

Studies on the role of cell division during neuronal differentiation in P19 EC cells

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Cell cycle progression must be tightly coordinated with cell fate choice. In this regard, cell division is one of the crucial factors that ensure differentiation process, as exemplified by mitotic clonal expansion during adipogenesis. However, there is not much of evidence and underlying mechanism that help us understand how cell division might play a role in other types of differentiation, ensuring controlled tissue development and homeostasis. Here, I focused on the involvement of cell division during neuronal differentiation. I used retinoic acid (RA)-induced *in vitro* neurogenesis system of P19 embryonic carcinoma cells to examine the direct link between cell division and neuronal differentiation. I observed that cell cycle blockers inhibited neuronal differentiation of P19 cells. In order to

investigate the underlying mechanisms, I screened for RA target genes whose transcripts were reduced with cell cycle blockers and identified *Tshz1* as a candidate for the cell division-dependent genes. The promoter analysis of *Tshz1* found the minimal essential region for RA and cell division-dependent transcriptional activation. Through computational sequence analysis of the promoter, E2F1 was predicted a possible upstream transcription factor for *Tshz1*. Furthermore, the E2F1 binding activity on the *Tshz1* promoter was reduced with the thymidine treatment. Taken together, E2F1 may function as a transcription factor whose activity is controlled in a cell division-specific manner for RA induction of *Tshz1* expression. This study is an example that cell division itself functions as a regulatory mechanism to ensure neuronal differentiation.

Keywords: Stem cell, Differentiation, Cell division, Retinoic acid, Transcription factor, Neurogenesis, P19 cell

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Background

Background

1. Cell cycle control during differentiation

Since the characterization and isolation of embryonic stem cells, understanding the intrinsic and extrinsic factors that control pluripotency of stem cells has been the core of stem cell research field. Although the major factors essential for pluripotency maintenance (i.e. Yamanaka factors) were discovered, it is still poorly understood as to which additional parameters are important for a specific cell fate acquisition. Cell cycle is a universal process that is displayed by almost all types of cells but its potential role during differentiation is only beginning to be revealed. Studying specific functions of cell cycle in the context of lineage-specification during differentiation may be a key to understanding how stem cells sense and decide to acquire its destined fate. In this section, the current understanding link between cell cycle and differentiation will be discussed.

1.1 Cell cycle structure of pluripotent vs. differentiated cells

It has been well reported that undifferentiated stem cells and terminally differentiated cells display distinct cell cycle profile. Undifferentiated stem cells divide much more rapidly (Hindley and Philpott, 2013), whereas, fully

differentiated cells usually exit the cell cycle from G1 to G0 and no longer proliferate. Despite much earlier observation, the precise kinetics behind the cell cycle change could not be investigated due to technical difficulty.

Recent innovation in scientific tool, called Fluorescent Ubiquitylation-based cell-cycle indicator (FUCCI), have allowed to directly test the sequential relationship between changes in cell cycle structure and differentiation (Sakaue-Sawano et al., 2008). Fluorescent fusion proteins of these molecules label cell cycle phase, and this tool allowed to measure individual cell's total cell cycle length and G1 phase length without artificially altering the cycle by pharmacological means. Using FUCCI as a tool, it is now well recognized that undifferentiated cells have much shorter cell cycle length, and differentiation signal quickly results in shorter cell cycle length, mainly due to increased G1 phase (Figure 1) (Roccio et al., 2013).

1.2 G1 phase during differentiation

In 1974, Pardee have implicated role of specific cell cycle phase that is required for differentiation. The physiological significance of G1 phase was implied by demonstrating the existence of Restriction point (R-point) as a time point when cell decides to exit the cell cycle or to complete the next cycle (Pardee et al., 1974). In 1985, Zetterberg and Larsson have developed

the importance of R-point further by showing its role in cell fate acquisition (Figure 2). The group have discovered that only the cells in early G1 phase exit the cell cycle and become quiescent upon serum deprivation (Zetterberg and Larsson, 1985).

In this view, main efforts were on understanding the link between cell cycle and differentiation through G1 phase, and now it is described as a window of opportunity for the cells to decide either to self-proliferate or to differentiate (Figure 2). A recent study on cell cycle machinery's role in cell fate acquisition provided a mechanistic clue on how G1 phase might affect differentiation. Cyclin D-CDK4/6 complex, which regulates G1 phase in conventional cell cycle, was found to be a player that also controls signaling molecules for lineage-specification at G1 phase (Pauklin and Vallier, 2013). The study explains that the early G1 phase is permissive for endoderm differentiation, while the late G1 phase is only permissive for neuroectoderm specification in hESCs (Vallier, 2015)

2. Cell division during differentiation

In this study, cell division itself was examined for potential regulator of differentiation. Due to the popular tendency for researchers to focus on G1 phase and its length when studying differentiation process, cell division's role was relatively overlooked. Nevertheless, the role of cell division has been implicated in differentiation of several systems, such as adipogenesis, myogenesis, and activation of specific gene-clusters.

2.1 Mitotic clonal expansion (MCE) during adipogenesis

Adipogenesis is a differentiation process by which mesenchymal stem cells become mature adipocyte containing large cytosolic lipid droplets. During *in vitro* differentiation of 3T3-L1 cells, cells firstly undergo contact inhibition and exit the cell cycle to G0, but treatment of cocktail of differentiation inducers drive the arrested cells to reenter the cell cycle (Figure 3). Several rounds of cell cycle during this process, called mitotic clonal expansion (MCE), was understood as an event that is associated with adipogenesis but the precise link was unclear.

To study the process, *in vitro* differentiation of preadipocyte cell line, 3T3-L1, was used, and the course of synchronous cell division was directly

monitored by time course analysis of total cell number and FACS analysis. Time course analysis of total cell number showed that the number doubled around 36 hours and quadrupled nearly 60 hours after growth arrest. This implied that cells divided once every 30 hours post growth arrest. In parallel, time course analysis of DNA content by FACS also showed similar result. DNA content was doubled synchronously, which was reduced by half about 30 hours after growth arrest. This synchronous cell cycle reentry and subsequent two to three rounds of cell cycle was called mitotic clonal expansion.

In an attempt to address the requirement of MCE for adipogenesis, the biological significance of MCE was confirmed by treatment of Cdk2 inhibitor, roscovitine, during MCE. The result showed reduced accumulation of cytoplasmic content of triacylglycerol and reduced expression of adipocyte markers (Tang et al., 2003). Therefore, in adipogenesis, the importance of MCE lied in ensuring functional differentiation of L1 cells into adipocytes because cell cycle block or inhibition of MCE resulted in inhibited adipogenesis. In fact, expression of essential upstream master regulators, such as, C/EBP beta, delta, alpha, and PPAR-gamma that control the expression of downstream adipocyte genes was known as the most direct way to control adipocyte differentiation. These studies suggest that MCE controls adipogenesis at an upstream level by acting as a licensing factor for

the progression of adipogenesis.

Time course analysis of EMSA assay using consensus C/EBP beta binding DNA sequence and nuclear extracts showed that C/EBP beta, one of the upstream regulators, is expressed early during differentiation, which acquire DNA binding activity after G1-S transition during MCE (Tang and Lane, 1999). DNA binding activity, which is essential for the expression of other important downstream factors, is only gained after C/EBP beta is phosphorylated by GSK-3beta and subsequently by Cdk2 (Li et al., 2007). In this respect, completion of cell divisions functions as licensing factor that allows for transcriptional cascade, which is required for completion of differentiation process.

2.2 Cell proliferation during myogenesis

Myogenesis is another differentiation process that results in development of muscle cells. In a study that aimed to find how myogenic activation by Lbx1 and Pax3 is controlled, it was discovered that overexpression of Lbx1 and Pax3 resulted in both increased cell proliferation and upregulation of their downstream myogenic targets. The importance of cell proliferation was suggested when blocking Lbx1 and Pax3-dependent cell proliferation resulted in a complete lack of myogenic markers MyoD, myogenin and myosin heavy chain despite overexpression of Lbx1 and Pax3

(Mennerich and Braun, 2001). Unfortunately, the study did not focus on further investigating molecular mechanism related to cell proliferation the reason why cell proliferation is needed during myogenesis needs to be further investigated. Nevertheless, it is still a good example that expression of master regulator is not sufficient for differentiation and that cell proliferation is also needed for the process.

2.3 DNA replication and temporal gene activation

As mentioned above, differentiation is an intricate process that requires not only the transcriptional activation of factors, but also a controlled interplay between these factors. To ensure the well-scheduled expression of factors, transcriptional activation of early developmental gene has been suggested to be linked to cell cycle through DNA replication. This potential link may explain how cells control sequential expression of development-related genes. *HoxB*, a homeobox gene that is important for body patterning, is expressed very early during development and their gene expression was observed to convey temporal collinearity in RA-induced P19 cells (Fischer and Mechali, 2003), which was abolished upon inhibition of DNA replication. This result showed that, in synchronized P19 cells, *HoxB* genes are linked to developmental transcriptional control through the cell cycle by

being activated within the first round of cell cycle, especially during S phase.

3. Neuronal differentiation of P19 cells

In this study, P19 cells were used as a model system to study the molecular mechanism underlying the role of cell division during neuronal differentiation. The main advantage of using P19 cells for this study lies in well-defined *in vitro* culture condition for efficient neuronal differentiation. Moreover, differentiation of P19 cells resembles that of early development. Treatment of retinoic acid in P19 cells not only results in functional neurons and glial cells, but also the differentiation adequately represents *in vivo* neurogenesis and gliogenesis process.

3.1 P19 embryonic carcinoma cells

P19 cell is murine embryonal carcinoma cell, pluripotent stem cells of malignant teratocarcinomas, which can efficiently differentiate into neurons in the presence of retinoic acid (RA). P19 cells were originally derived from a teratocarcinoma, which was formed by transplanting C3H/He mouse embryo into a host mouse testis (McBurney MW and Rogers BJ, 1982). With appropriate culture condition, P19 cells can be induced to differentiate into embryonic body (EB) with cell types of three germ layers (ectoderm, endoderm, or mesoderm). Because of its well characterized condition for specific type of differentiation, well-representation of *in vivo* early

differentiation process, and relatively convenient in vitro culture system, it is widely used as a useful system to study differentiation in vitro (Jones-Villeneuve et al., 1982).

3.2 Retinoic acid-induced transcription

Retinoic acid (RA) is one of the major vertebrate lipophilic morphogens that is essential for the correct patterning during early embryogenesis. It is known that RA controls expression of gene cluster known as homeobox genes during anterior-posterior (A-P) patterning (Simeone, A. et al, 1995). RA is signaling molecule that exerts its action through different types of retinoic acid receptors (RARs). These ligand-dependent transcription factors then act in heterodimeric combinations with one of the retinoid X receptors, which together bind to specific DNA sequence of RA-reponse elements (RAREs) (Niederreither and Dollé, 2008). (Figure 4)

3.3 Studying the role of cell division in P19 cells

Previous studies have shown that RA-induction in P19 cells occurs mainly in G1 phase. However, the study also implied that RA-induction at G1 phase alone is insufficient for commitment to occur, which was also demonstrated using mouse embryonic stem cells (ESCs) (Li et al., 2012). In

RA-induced differentiation, P19 cells need at least 24 hours to fully commit to differentiate and few rounds of cell cycle were implicated as important events (Mummery et al., 1987).

Despite many additional studies on roles of cell cycle in stem cell differentiation, it is still unclear as to why certain events are necessary during the initial 24 hours after RA induction. Therefore, this study attempts to answer the unexplored question on the requirement of several rounds of cell cycle during early hours of RA treatment for cells to differentiate. In this study, the main goal is to investigate role of cell division during RA-induced neuronal differentiation of P19 cells. Identification of specific cell division-dependent differentiation regulator will open a new doorway to understand cell division-dependent regulation of stem cell differentiation.

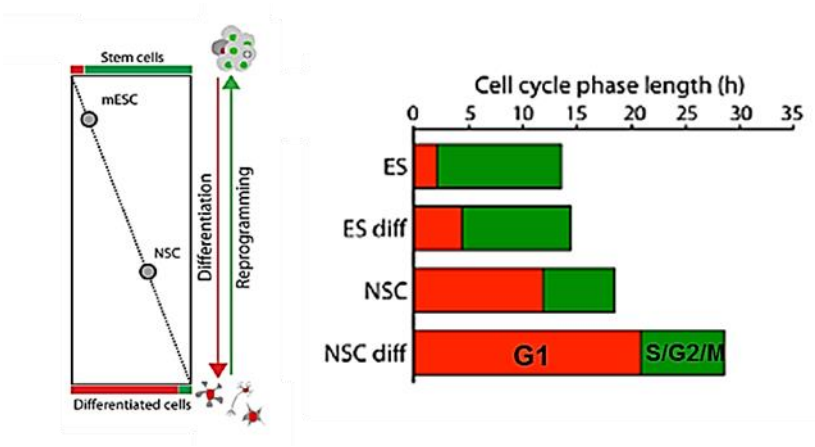

Figure 1. G1 phase lengthening upon differentiation. During differentiation, cell cycle characteristics of stem cells undergo drastic changes. Differentiation signal-induced cells acquire much longer G1 phase (labeled in red color), resulting in an increased total cell cycle length (Roccio et al., 2013).

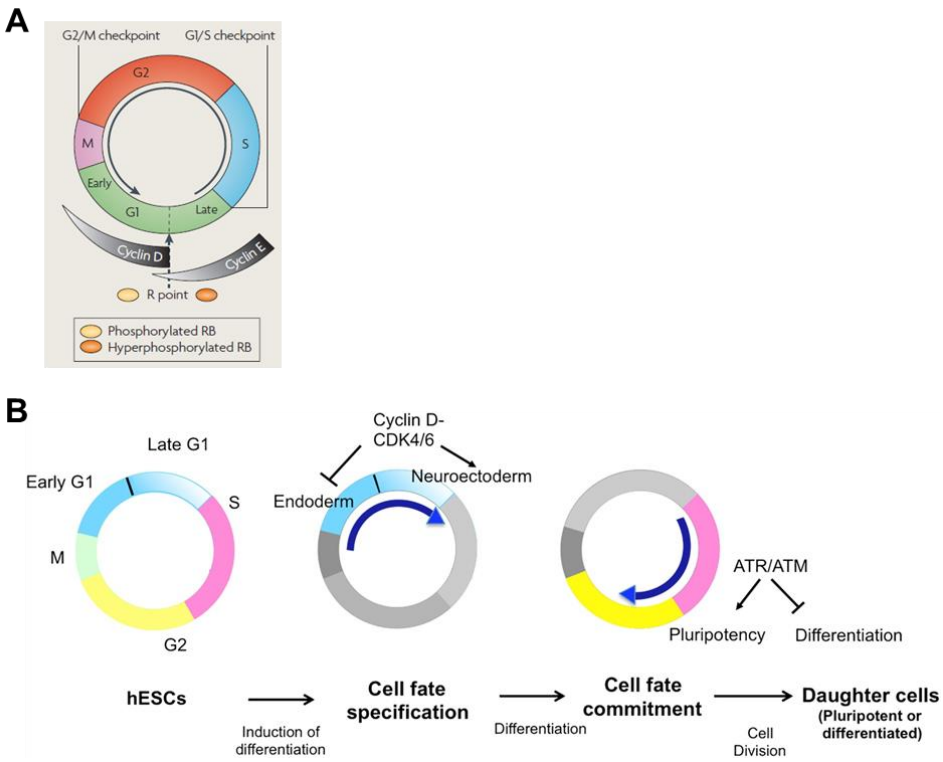

Figure 2. Current model on how cell cycle drives differentiation of stem cells. (A) G1 phase is characterized by Restriction point (R-point). R-point divides G1 phase into early and late G1 phases and marks a point of no return for the cells. Once cells pass through R-point of G1 phase, cells are committed to complete the cycle (Orford and Scadden, 2008). **(B)** Recent studies show how lineage specification occurs during G1 phase. Early G1 is responsible for endoderm and late G1 is responsible for neuroectoderm lineage specification. Following studies also suggested that subsequent cell cycle phase, G2 phase, drives the cells to lose pluripotency (Vallier, 2015).

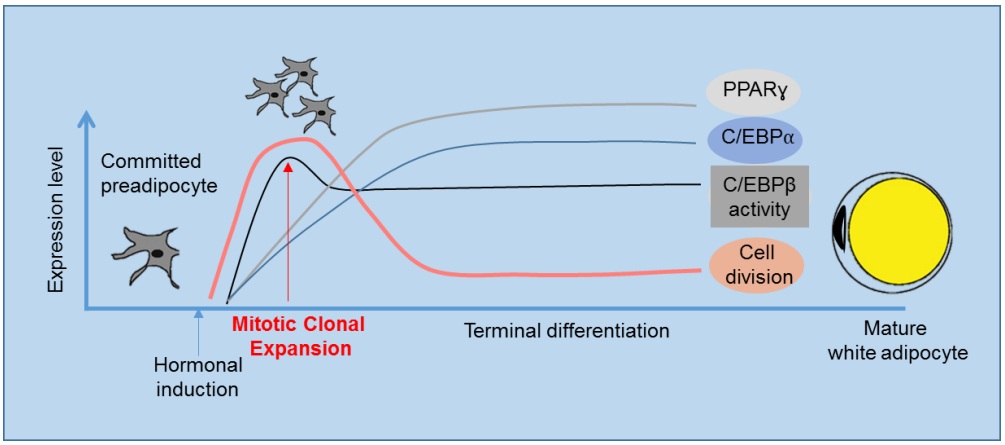

Figure 3. Mitotic Clonal Expansion (MCE) during adipocyte differentiation. During adipogenesis, cell cycle-arrested cells undergo synchronous cell division known as MCE. This process is understood as licensing process for cells to complete adipogenesis. (modified from Tang and Lane, 2012).

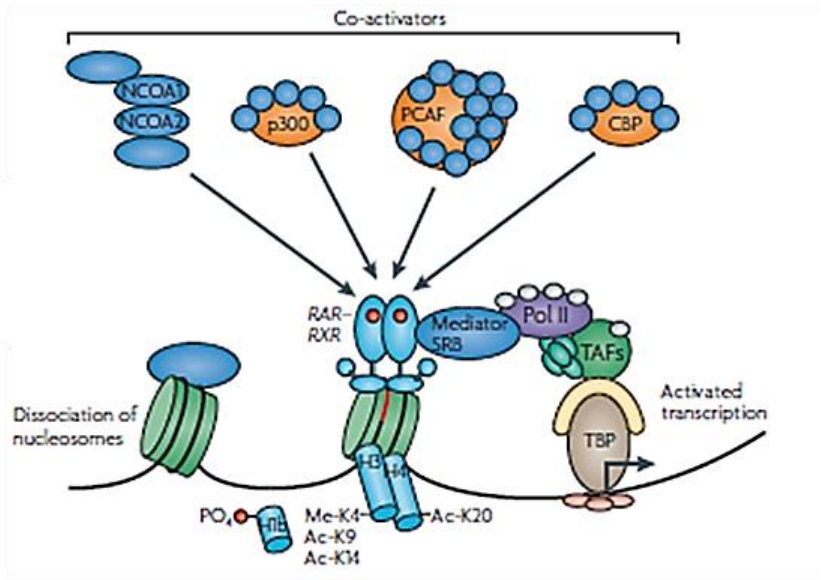

Figure 4. RA-dependent transcriptional control. RA acts as a ligand for several nuclear receptors, combinations of which form heterodimers. The complex then binds to a RARE (RA-response element), thereby directly controlling the transcriptional activity of target gene. (Niederreither and Dolle, 2008).

Abstract

Abstract

Cell cycle progression must be tightly coordinated with cell fate choice. In this regard, cell division is one of the crucial factors that ensure differentiation process, as exemplified by mitotic clonal expansion during adipogenesis. However, there is not much of evidence that the cell division also plays a role in other types of differentiation. Here, I focused on the involvement of cell division during neuronal differentiation. I used retinoic acid (RA)-induced in vitro neurogenesis system of P19 embryonic carcinoma cells to examine the direct link between cell division and neuronal differentiation. I observed that cell cycle blockers, such as thymidine and RO3306, inhibited neuronal differentiation of P19 cells. In order to investigate the underlying mechanisms, I screened for RA target genes whose transcripts were reduced with cell cycle blockers and identified *Tshz1* as a candidate for the cell division-dependent genes. The promoter analysis of *Tshz1* found that the 50 bp region at -1250/-1200 are minimal essential region for RA and cell division-dependent transcriptional activation. Through computational sequence analysis of the promoter, E2F1 was predicted a possible upstream transcription factor for *Tshz1*. In fact, endogenous *Tshz1* transcript levels were enhance in E2F1-overexpressing P19 cells, whereas they were reduced in the E2F1-depleted cells. Specific

binding of E2F1 on the *Tshz1* promoter was confirmed with the electrophoretic mobility shift and chromatin immunoprecipitation analyses. Furthermore, the E2F1 binding activity on the *Tshz1* promoter was reduced with the thymidine treatment. Taken together, E2F1 may function as a transcription factor whose activity is controlled in a cell division-specific manner for RA induction of *Tshz1* expression. This study is an example that cell division itself functions as a regulatory mechanism to ensure neuronal differentiation.

Introduction

Introduction

Differentiation is an intricate process by which uncommitted cells acquire its cell fate. It has been widely accepted that expression of key master regulators (i.e. transcription factor) or external stimuli are common ways for stem cells to differentiate but stochastic differentiation and heterogeneous cell fate of stem cell could not be explained. Recently, unusual mode of cell cycle regulation of stem cells became the main focus in the research field. Cells undergoing differentiation display distinct cell cycle structure of lengthened G1 phase and no longer divide after terminal differentiation (Coronado et al., 2013; Hindley and Philpott, 2013). Many studies were based on the idea that G1 phase may act as a gateway to integrate differentiation signal into cell fate acquisition (Coronado et al., 2013; Pauklin and Vallier, 2013; Pauklin et al., 2015; Singh et al., 2013). Despite the importance of a specific cell cycle phase on the induction of differentiation signal, lengthening G1 phase is not sufficient for the induction of differentiation (Li et al., 2012). In RA-induced differentiation, P19 cells need at least 24 hours to fully commit to differentiate and several rounds of cell cycle were implicated as important events (Mummery et al., 1987). In this aspect, cell division is not a mere way for cells to expand but another potentially important event that regulates differentiation as seen in

adipogenesis, myogenesis, and early embryo development, but the underlying molecular mechanisms has not been fully investigated.

Several lines of evidence support the link between cell division and differentiation. Cell division is a common process by which mother cell gives rise to genetically identical two daughter cells. On a multicellular level, the role of cell division is much more complex as it occurs not just to produce clones but to organize the tissue. It is frequently adopted to produce more specialized cells, such as gametes. Through cell division, cells collectively control cell growth, cell morphology, as well as tissue development.

The role of cell division during differentiation has been implicated in tissue development. In tissues, spindle assembly and morphology are coordinated to control mode of cell division so that specific cell types are made (Dudka and Meraldi, 2017). During development of cerebral cortex, cells need to be both expanded and differentiated to reserve specific cell population. Stem cells undergo specialized division known as symmetric self-renewing division and asymmetric neurogenic division to maintain a pool of both neural stem cells and neural progenitor cells. While symmetric division results in expansion of neural stem cells, they later undergo asymmetric division. One of the daughter cells remains a proliferating progenitor and the other become neuron after one or multiple rounds of

transit amplifying divisions (Homem et al., 2015). In this way, early proliferative divisions are necessary for the expansion of progenitor pool, and the timing of the switch to later asymmetric or symmetric divisions determines the development of different regions of the brain (Hardwick and Philpott, 2014). In this way, role of cell division is tightly linked to a normal development of tissue, thus, homeostasis of an organism.

In other cases, cell division acts as a way to directly regulate differentiation process by controlling the expression of lineage specific genes. Mitotic clonal expansion (MCE) is a type of specialized cell division that is known as a prerequisite for adipocyte differentiation (Tang and Lane, 2012). MCE is required for activation of C/EBPß and expression of C/EBPα and PPARγ, both of which are essential for ensuring adipocyte differentiation. MCE is two to three rounds of synchronous cell division that sequentially take place upon hormonal induction, which signals the onset of adipogenesis. It functions as licensing factor that allows for transcriptional cascade, which is required for the completion of adipocyte differentiation process (Tang et al., 2003). Similar function of cell division is also reported relating transcriptional regulation of *Hox* family gene which is known as important factor during early development. *HoxB* body pattern-related genes are linked to developmental transcriptional control through the cell division by being activated within the first round of cell cycle after differentiation (Fisher and

Mechali, 2003). These controlled differentiation processes partially explain why terminal cell fate acquired cells no longer divide. Nevertheless, these still do not explain why differentiating cells need to undergo multiple rounds of cell division before acquiring its final state of differentiation.

Heterogeneity in pluripotent stem cells is repeatedly observed but its nature has not been fully understood (Singh et al., 2013). Because stem cells have particularly high heterogeneity on a population level, it is difficult to examine a single mechanism that collectively controls gene expression during differentiation. In order to decipher the direct link between cell division and differentiation, a single population of cell line can be a useful tool. As shown by Sawano and his colleagues who analyzed cell cycle of developing neural tissues using Fucci transgenic mice, cells in brain tissue display spatiotemporal dynamics (Sakaue-Sawano et al., 2008). Despite the powerful images of the link between cell cycle and differentiation *in vivo*, there is still a lack of mechanistic understanding at molecular level.

P19 cell is murine embryonal carcinoma cell, pluripotent stem cells of malignant teratocarcinomas, which can efficiently differentiate into neurons in the presence of retinoic acid (RA). It is widely used as a convenient system to study differentiation *in vitro,*which sufficiently represents *in vivo* neurogenesis process of mouse brain (Jones-Villeneuve et al., 1982). Previous studies have shown that RA-induction in P19 cells

occurs mainly in G1 phase. However, the study implied that RA-induction at G1 phase alone is insufficient for commitment to occur, which was also demonstrated using mouse embryonic stem cells (mESCs) (Li et al., 2012). Despite many additional studies on the role of cell cycle in stem cell differentiation, it is still unclear as to why certain events are necessary during the initial 24 hours after RA induction. To help understand this process, I looked carefully into the old paper (Mummery et al., 1987) and revisited the question on the requirement of several rounds of cell divisions during early hours of RA induction for cells to differentiate into neurons.

In this study, I investigated the role of cell division during RA-induced neuronal differentiation of P19 cells. Time-course cell cycle analysis and neurite outgrowth assay implied the requirement of cell division during early hours of neuronal differentiation for neurogenesis and neurite formation. Furthermore, screening for cell division-dependent genes among RA-induced genes by RT-PCR identified several genes whose expressions are reduced upon inhibition of cell division. Among the candidates, Tshz1, a DNA binding zinc finger protein, was selected for in-depth study. Promoter analysis of *Tshz1* revealed a specific region that is necessary for RA and cell division-dependent transcriptional regulation. Furthermore, using sequence specificity results from EMSA mutation assays and computational analysis, E2F1 was examined as a potential upstream transcription regulator that

activates *Tshz1* transcription in a RA and cell division-dependent manner. EMSA supershift assay, E2F1 overexpression followed by *Tshz1* transcription analysis, and ChIP assays using anti-E2F1 support that E2F1 may positively regulate *Tshz1* transcription in RA-induced P19 cells. To my knowledge, this is the first evidence directly linking cell division to differentiation and identification of specific cell division-dependent differentiation regulator will open a new doorway to understand cell division-dependent regulation of stem cell differentiation.

Materials and Methods

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Antibodies

The antibodies against Tuj1 (Covance, MMS-435p-100), phospho-histone-H3 (Upstate), p27 (Abcam, ab7961), E2F1 (Santa Cruz Biotechnology, sc-251), GFP (Santa Cruz Biotechnology, sc-9996), Flag (Sigma-Aldrich, F3165) GAPDH (Ambion, AM4300) were commercially purchased. The Alexa-fluorescence secondary antibodies were purchased from Invitrogen. The mouse and rabbit IgG-HRP antibodies were purchased from Sigma and Millipore, respectively.

Cell culture and drug treatment

P19 embryonic carcinoma cells were cultured in DMEM (Welgene) supplemented with 10% FBS and antibiotics (5 μg/ml ANT-MPT; InvivoGen) at 37˚C and 5% CO_2. The single thymidine block method was used to synchronize the cell cycle. The cells were treated with 2 mM of thymidine for 12 h and released after cells were PBS washed at least twice. For G2 arrest, the cells were treated with RO3306 (10 μM) for 12 h.

For depletion of endogenous E2F1 and Tshz1, *siE2F1*-1 (5′-CGC UAU GAA ACC UCA CUA ATT-3′) and *siE2F1*-2 (5′-GUG GAU UCU UCA GAG ACA UTT-3′) and *siE2F1*-3 (5′-GAG GGC AUU AGA GAU

CUC UTT-3′) and *siTshz1* (5′-CCC AGA UAC UCA AGU GCA UTT-3′) were used. A scrambled siRNA sequence (*siCTL*; 5′-UUC UCC GAA CGU GUC ACG UTT-3′) was used as a control. RNAiMAX (Invitrogen, 13778-075) was used for siRNA transfection according to the manufacturer's protocol.

In vitro neuronal differentiation

The conventional method was performed essentially according to the previous report (McBurney et al., 1988). Briefly, P19 cells (1.0 × 105 cells/mL) were cultured in suspension in a Petri dish in DMEM containing and treated with 1μM all trans-retinoic acid (R2656 Sigma) to allow the development of embryonic bodies (EBs). The EBs were then trypsinized and replated into a poly-l-lysine-coated tissue culture dish and cultured in FBS-free B27-supplemented DMEM medium (Gibco).

Immunocytochemistry analysis

P19 cells were seeded on cover glass were fixed with cold methanol for 10 min and washed three times with cold PBS. After incubation of PBST (0.1% Triton X-100 in PBS) for 10 min, the cells were blocked with blocking solution (3% bovine serum albumin, and 0.3% Triton X-100 in PBS)

for 30 min. Then cells were incubated with primary antibodies diluted in blocking solution for 1 h, washed three times with PBST, incubated with secondary antibodies in blocking solution for 30 min. Afterwards, cells were washed twice with PBST, incubated with 4,6-diamidino-2-phenylindole (DAPI) solution for 3 min and washed twice with PBST. The cover glasses were mounted on a slide glass with ProLong Gold antifade reagent (Life Technologies, P36930). Images were acquired from fluorescence microscopies equipped with digital cameras (Olympus IX51 equipped with QImaging QICAM Fast 1394 or Olympus IX81 equipped with ANDOR iXonEM+) and processed in ImagePro 5.0 (Media Cybernetics). Inset images were enlarged four times in Image J 1.49 (National Institutes of Health)

Immunoblot analysis

Cells were lysed on ice with RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% SDS, 50 mM Tris-HCl at pH 8.0, 10 mM NaF, 1 mM Na3VO4, 1 mM EDTA and 1 mM EGTA) containing a protease inhibitor cocktail (Sigma-Aldrich, P8340). The cell lysates (30 μg protein) were loaded onto a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (GE healthcare).

RT-PCR

Total RNAs were extracted using Trizol (Invitrogen) and reverse transcription was performed from 2 μg total RNAs using RnaUsScript RT (LeGene). The abundance of mRNA was detected by an ABI prism 7500 system with SYBR TOPreal qPCR 2× PreMix (Enzynomics). The quantity of mRNA was calculated using the ΔΔCt method and Beta-actin, Gapdh and Hmbs were used as control.

Luciferase assay

A promoter reporter construct containing -1500 to +500 relative to TSS of *Tshz1* promoter was inserted upstream of the Firefly luciferase reporter in the pGL3-Basic vector (Promega). To make stable cell line of

luciferase reporter, neomycin (kanamycin) - expressing sequence was inserted. 24 h after transfection of these constructs, P19 cells were subjected to G418 selection for 1 week. Then, polyclonal cell lines were subjected to luciferase assays using microplate luminometer (DE/LB 96V, Berthold Technologies). For construction of *Tshz1* mutants, site-directed mutagenesis was performed. Luciferase outputs were normalized by total protein amount measured by Bradford assay. All values were calculated as relative to pGL3 vector.

EMSA assay

The oligonucleotides were first biotinylated and annealed, using the Biotin 3'End DNA labeling Kit (Thermo Fisher Scientific, Rockford IL, USA). Nuclear extracts were prepared from P19 cells using nuclear extraction buffer. Binding reactions were performed using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Rockford, IL, USA). The reactions were performed on ice for 20 min with nuclear extract and 10 fmol of labeled DNA in a final volume of 20 μl, containing 2 μl of 10x binding buffer, and 1 μl of Poly (dI.dC). For supershift assays, 1-3 μg of E2F1 antibody (Santa Cruz Biotechnology) was added to the reaction mixture described above, followed by incubation on ice for 15 min prior to

the addition of the labeled oligonucleotide probe. For all the supershift assays, DTT-free buffers were used. The resultant DNA-protein complex was resolved from free oligonucleotide in a 6% native polyacrylamide gel in Tris borate–EDTA buffer, and transferred to nylon membranes (Biorad). The membranes were cross-linked by UV radiation using Gel-doc (Bio-Rad Laboratories). Biotin signals were detected using a Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. A double-stranded mutated oligonucleotide and unlabeled double stranded oligonucleotides were used to examine the specificity of DNA binding.

FACS analysis

Samples were collected over indicated time points and fixed in 70% ethanol overnight. For cell cycle analysis, fixed cells were treated with RNase for 20 minutes before addition of 5μg/mL propidium iodide (PI) and analyzed by FACS (BD Biosciences FACScalibur).

Chromatin immunoprecipitation assay

P19 cells were crosslinked with 13% formaldehyde for 10 min at room temperature. After glycine quenching, the cell pellets were lysed in

buffer containing 50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS, supplemented with complete protease inhibitor cocktail (Roche), and sonicated. Chromatin extracts containing DNA fragments with an average of 250 bp were then diluted ten times with dilution buffer containing 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl (pH 8.1) with complete protease inhibitor cocktail, pre-cleared with protein A/G sepharose and subjected to immunoprecipitations overnight at 4℃. Immunocomplexes were captured by incubating 45 µl of protein A/G sepharose for 2 h at 4℃. Beads were washed with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl), buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 10mM Tris-HCl (pH 8.1), 1mM EDTA), TE buffer (10mM Tris-HCl (pH 8.0), 0.5M EDTA) and eluted in elution buffer (1% SDS, 0.1 M NaHCO3). The supernatant was incubated overnight at 65℃ to reverse-crosslink, and then digested with RNase A for 2h at 37℃ and proteinase K for 2h at 55℃. ChIP and input DNA were then purified and analysed for PCR analysis.

Neurite outgrowth assay

Typically, pictures of 30-50 neurons from three separate coverslips from each experiment were taken using a fluorescent microscope (Leica

DMI6000B). Representative cells with strong Tuj1 immunoreactivity labeling neurite (axonal and dendritic) processes were analyzed. Neurites that had lengths that were at least twice the diameter of the cell body were measured. Neurite lengths from the cell body and cell body diameter were traced and measured using Neuron J (Image J) software and the data were compiled and analyzed using Prism (GraphPad).

Statistical Analysis

All experiments were performed independently at least three times. Values are expressed as mean±S.D. Significance was analyzed using two-tailed, unpaired t-test, and one-way ANOVA. $p<0.05$ was considered statistically significant.

Results

Results

Cell division control at an early stage of neuronal differentiation in P19 cells

To study the role of cell division during differentiation, I examined if there is a time-dependent changes in cell division rate during neuronal differentiation. First, time-course analysis of cell division after RA-induction was performed. To do this, P19 cells were suspension cultured with RA to form embryonic body (EB) and the total cell number was quantified using hemocytometer every 24 hours for 4 days (Figure 5A). The result showed that total cell number increased with time, indicating that majority of cells actively divide until 48 hours after RA induction. Then, total cell number plateaued from 72 hours with a slight decrease at 96 hours time-point but the reduction level was not statistically significant. Next, percentage of mitotic cells were quantified by immunostaining for phosphorylated histone H3-positive cells (Figure 5B) to see if similar time point change in number of mitotic cells correlates with the above result. The result showed that 30% of cells undergo mitosis until 48 hours post RA induction, after which decreased to about 10%. Together, these results show that cells actively divide until 48 hours and expanding cell numbers decrease from around 48 hours after RA induction in P19 cells.

To understand the overall pluripotent and proneural state during neuronal differentiation, P19 cells were treated with RA and induced to form EB for 4 days. Then, expression level of pluripotency markers, *Oct3/4* and *Sox2*, proneural markers, *Nestin* and *Ascl1* were measured. The result showed that pluripotency is lost quickly, within 24 hours of RA treatment (Figure 6A,B), whereas proneural markers are upregulated gradually and relatively later during RA-induction (Figure 6C,D). Overall, the result indicated that cells are gradually undergoing neuronal differentiation.

As reported by many researchers, one of the distinct features of cells undergoing differentiation is a dramatic increase in G1 phase length (Roccio et al., 2013). To see if decrease in cell division rate is accompanied by altered cell cycle structure, time-course cell cycle profile was analyzed using FACS analysis (Figure 7A). FACS result showed that G1 phase cells increase after RA-induction, whereas S phase and G2/M phase cells decrease overtime. Cells in G1 phase dramatically increased 48 hours post RA induction, which is similar time point to the change in total cell number (Figure 7A).

It is well known that cells arrested at G1 phase exit to G0 phase in order to terminally differentiate (Cheung and Rando, 2013). Tumor suppressor $p27^{Kip1}$ is one of the natural CDK inhibitors that negatively regulates cell cycle progression at G1 phase. It has been previously reported

that $p27^{Kip1}$ is a key mediator of growth arrest in RA-induced neuronal differentiation using other cell lines (Matsuo and Thiele, 1998; Cuende et al., 2008). To see if increase in G1 phase cells represents an increase in cells undergoing cell cycle arrest, protein level of stable $p27^{Kip1}$ was measured by immunoblot (Figure 7B). The protein level of $p27^{Kip1}$ showed that there is a dramatic increase of cells undergoing G1 arrest, which occurs soon after the time-point when G1 phase cells start to increase. Taken together, these results imply that, after RA-induction, cell divisions gradually slow down at an early stage of neuronal differentiation in P19 cells.

Importance of cell division at an early stage of differentiation for neuronal differentiation and neuron morphogenesis

Having seen the change in cell division rate followed by increase in G1 length and cell cycle arrest during neuronal differentiation, I next investigated to see if this change was also linked to neuron formation and maturation. To do this, a time-course analysis of neurite outgrowth was performed to check if changes in cell cycle dynamics affect the cells final state of neuronal differentiation and neuron maturation. Neurite outgrowth measurement is way by which neuron formation is assessed. It involves a quantification of neurite formation that can be done by measuring neurite

length and number of branches (Filous and Silver, 2016). First, cells were treated with RA for different number of days. Then, cells were enforced to undergo neurogenesis by forming extended neurites. Using neuron-specific ßⅢ-tubulin Tuj1 as neuron marker, neurite outgrowth assay of Tuj1-positive neurons was quantified using Neuron J (Figure 8).

The immunostaining result of Tuj1-positive neurons showed that very few cells form extended neurites and robust neurite formation is seen from 48 hours post RA-induction (Figure 9). In order to see in detail if this affects not only neuron formation, but also neuron maturation, neurite length and branching were also quantified. The results showed that percentage of cells with primary neurites and neurite length dramatically increased from 48 hours after RA-induction (Figure 10A,B). Further analysis of neurites showed that longer hours post RA-induction resulted in similar neurite length but more mature neurons with extended branching (Figure 10). Along with time-course cell cycle dynamics analysis, these results implied that sufficient number of cell divisions during early hours post RA-induction may be important for cells to undergo neurogenesis and neuron maturation.

To directly test the requirement of cell division for neuronal differentiation, cell division was blocked by thymidine treatment, a cell cycle blocking drug, along with RA. After 48 hours of RA-treatment, cells were enforced to undergo neurogenesis. After 3 days of neuronal differentiation,

cells were analyzed by neurite outgrowth assay (Figure 11). The result showed a dramatic decrease in number of cells with neurites. This indicates that cell divisions during early stages of neuronal differentiation are important for neuronal morphogenesis. Overall, these results implied a strong link between cell division during 48 hours post RA induction and neuronal differentiation (Figure 12).

Identification of RA-induced genes that are regulated in a cell division-dependent manner in P19 cells

In order to understand how cell division could control differentiation, mechanistic study was performed. One of the common ways by which cells control differentiation is transcriptional regulation of lineage-specific genes. Therefore, I set up an experimental scheme to see if cell division could regulate differentiation process by transcriptional regulation. To test this idea, a list of RA-induced genes was made based on the data from previously published papers (Tanoury et al., 2014; Mahony et al., 2011; Balmer and Blomhoff, 2002), and 38 candidate genes were tested for cell division-dependency.

First, to confirm their RA-induced transcriptions, cells were treated with or without RA and mRNA expression was analyzed via RT (Reverse

Transcription) -PCR. Thymidine was treated along with RA to see the effect of blocking cell division on transcription and mRNA level was measured in parallel (Figure 13). The RT-PCR result showed that most of the genes tested were strongly induced by RA in P19 cells, confirming that referenced papers data indeed include legitimate RA target genes (Figure 14). Also, most of the genes showed strong transcriptional induction within 24 hours of RA treatment, however, the induced genes may be RA direct or indirect target genes. Next, to test for cell division-dependency, P19 cells were also treated with thymidine to block cell division (Figure 14). For the subsequent in-depth study, 6 genes were selected *out* as they were up-regulated by thymidine treatment alone and were involved in RA metabolism. Finally, the results identified 11 cell division-dependent genes whose expressions were decreased with the thymidine treatment (Figure 14B). These candidates were further categorized to select for the gene that is suitable for further studies and, as a result, *Tshz1* was selected.

To further confirm RA-induced *Tshz1* expression with or without cell division, qPCR was performed using two different types of cell cycle blocking drugs, thymidine and RO3306 (CDK1 inhibitor, G2/M phase arresting drug) (Figure 15B). The result was consistent with the RT-PCR result and *Tshz1* expression was reduced by half when treated with either thymidine or RO3306. To see the cell division-dependent expression pattern

of *Tshz1* more in detail, time-course analysis *Tshz1* mRNA was performed by synchronizing cells at G1/S phase (Figure 14A,C). RA was treated upon cell cycle release and samples were collected every 3-6 hours. The result showed that *Tshz1* expression is strongly induced soon after first round of cell cycle after RA treatment, considering that P19 cell's doubling time is about 12 hours (Figure 15B). This expression pattern also suggests that RA-induced *Tshz1* expression is linked to cell division.

Tshz1 is a transcription factor with zinc finger motif. Although Tshz1 had been known to be involved in trunk patterning during *Drosophila* development, in mouse, it was suggested that it is required for olfactory bulb neurogenesis (Ragancokova et al., 2014). Conditional *Tshz1* KO mice in central and peripheral nervous system showed that it results in deficient sense of smell due to inability for progenitor cells to exit the cell cycle to become post-mitotic neuron. Since RA-induced differentiation of P19 cells is a well-known condition for neuronal differentiation (Jones-Villeneuve et al., 1982), these previous reports suggest that Tshz1 might also be involved in neurogenesis of RA-induced P19 cells. If cell division-dependent *Tshz1* expression upon RA-induction is one of important signaling pathways linking cell division to neuronal differentiation, RA-induced *Tshz1* expression itself could be important for neurogenesis. To test this possibility, Tshz1 was depleted using siRNA targeting *Tshz1* mRNA and cells were

induced to undergo neurogenesis (Figure 16A). Neurite outgrowth assay showed that *Tshz1* depletion (Figure 16B) resulted in reduced percentage of cells with primary neurites (Figure 16C,D). Although the reduced level was not dramatic, comparison with the control group was statistically significant, suggesting that expression of Tshz1 might be necessary, but not sufficient for neuronal differentiation.

Identification of *Tshz1* promoter region that is important for RA and cell division-dependent activation

So far, qPCR and RT-PCR results consistently showed that *Tshz1* expression is induced by RA, which is reduced when cell division is blocked. However, it is still unknown how this phenomenon is occurring at molecular level. In order to understand how *Tshz1* expression level is being reduced upon treatment of cell cycle blocking drugs, promoter region of *Tshz1* gene was cloned from P19 cells (Figure 17A) and analyzed using luciferase assay system (Figure 17B).

To reconfirm previous qPCR result, luciferase vectors were stably expressed in P19 cells. Then, the cells were treated with RA for 24 hours and sampled to measure luciferase activity as an indication of promoter activity. Luciferase assay result showed that -1500 bp to +500 bp relative to TSS

(transcription start site) region of *Tshz1* promoter is strongly activated by RA (Figure 17B). Consistent with qPCR result, transcriptional activity was reduced by half when cell division was blocked by thymidine treatment (Figure 17B). This result suggested that promoter activity of *Tshz1* gene can be activated by RA and may be regulated in a cell division-dependent manner.

Next, to further identify minimal essential region of *Tshz1* promoter that is responsible for cell division-dependent and RA-induced transcription, serial deletion mutants were constructed and analyzed using the same luciferase assay (Figure 18A). The result indicated that there is still a strong activity with -1300 to +500 bp promoter region but a dramatic decrease in promoter activity was observed when there was only -1100 to +500 bp region. To see if presence of -1400 to -1200 bp regions is sufficient for promoter activity, fragments of *Tshz1* promoter were fused to TK (thymidine kinase) promoter and luciferase assay was performed (Figure 18B). Series of assays using additional mutant constructions indicates that main essential region seems to be present within -1250 to -1200 bp of promoter region. Furthermore, -1250 to -1200 bp of promoter region alone was sufficient to be activated by RA, and cell division block also reduced promoter activity of this minimal region by half (Figure 19). These implied that -1250 to -1200 bp region is responsible for RA-induced transcription and its transcription is

being controlled via cell division-dependent pathway.

Putative transcription factors may bind to the -1250/-1226 sequence of *Tshz1* promoter in a RA and cell division-dependent manner

In order to further elucidate cell division-dependent control of neuronal differentiation, the next step was to search for a transcription factor that directly links cell division to neuronal differentiation by controlling *Tshz1* transcription at an upstream level. Having identified the minimal essential region of *Tshz1* promoter during RA-induced transcriptional activation, it was hypothesized that there is a factor that binds to -1250 to -1200 bp region in a nucleotide sequence-specific and cell division-dependent manner upon RA-treatment.

First, to test this possibility, Electrophoretic Mobility Shift Assay (EMSA) using P19 cells protein lysates and biotinylated -1250 /-1200 bp probe was performed to see if there is a candidate nuclear protein binding to the region upon RA treatment in a cell division-dependent manner (Figure 20). The EMSA result showed a strong retarded band, an indication of protein binding to the probe, only in a group mixed with RA-treated nuclear extract (Figure 20A). This band intensity increased in a nuclear protein

concentration-dependent manner (Figure 20B), indicating that there is nuclear protein binding to the probe in RA-dependent manner. Next, to confirm cell division-dependent binding, the probe was incubated with RA-treated nuclear extracts in the presence of thymidine (Figure 20C). The result was consistent with qPCR and luciferase assay in that the retarded band in RA-treated group disappeared when mixed with extract from cells whose cell division was blocked by thymidine.

To identify candidate transcription factor, it was necessary to further minimize the binding region. The 50 bp region was sub-divided into two regions and EMSA assay was performed to see if the binding occurs in a specific 25 bp region. The result showed that the binding occurs only in a -1250/-1225 region (Figure 21A). Using this region, time-course EMSA assay was performed to see if the RA-induced binding occurs in a cell-division dependent manner (Figure 21B). Consistent with the time-course *Tshz1* qPCR result, the binding band was started to be observed from 18 hours post RA-treatment, implying the binding is not only RA-dependent, but also requires cells to undergo at least one round of cell division.

Next, sequence of -1250/-1200 region was analyzed to make a list of possible binding transcription factors using PROMO3.0 computational analysis. The prediction list contained about 17 candidates. Interestingly, the consensus binding sequences of the candidates shared three common regions

within -1250/-1200 sequence. Therefore, these three regions (A, B, and C) common sequences were mutated (purine to pyrimidine) and the mutants were tested for nuclear protein-binding ability using EMSA assay (Figure 22A). The results showed that probe with region A mutation and region B mutation both failed to show shifted band but region C mutation still showed binding band, meaning regions A and B are important for binding with candidate nuclear factors (Figure 22B,C). Furthermore, *Tshz1* promoter -1250/-1200 containing the mutation was tested for promoter activity via luciferase assay (Figure 22D) and the result showed decreased activity that was reduced by half compared to the wildtype. From these results, I decided to focus on the candidates that were predicted to bind to these two regions.

E2F1 may be transcription factor that regulates *Tshz1* expression in a RA and cell division-dependent manner in P19 cells

Mutation analysis using EMSA assay showed that GCCGCG sequence within -1250/-1200 region is important for candidate binding. Recently, similar approach of promoter analysis and identification of binding factor study was published. The study showed that CCGGGCGGCCGG sequence of *HOXB9* promoter is responsible for transcriptional activation by

E2F1 (Zhussupova et al., 2014). Because these two sequences were similar to each other, E2F1 was chosen as a candidate among the list. E2F1, a transcriptional activator important for progression through the G1/S transition, was also functionally most linked to cell division among the list since it is known to be required for cell proliferation (Wu et al., 2001). Several members of E2F family were known to be required for other types of differentiation, such as adipogenesis (Fajas et al., 2002). First, the binding ability of E2F1 to *Tshz1* promoter's regulatory region -1250/-1200 was tested *in vitro* via EMSA supershift assay using E2F1 antibody (Figure 23). When RA-treated nuclear extract and probe were mixed with monoclonal antibody specific to E2F1, a strong supershift band was observed, implying that E2F1 might be one of the binding proteins present in the RA-treated nuclear extract.

Next, it was tested whether E2F1 is a transcription factor that activates endogenous *Tshz1* expression in a RA-dependent manner. To do this, mouse E2F1 was overexpressed in P19 cells and endogenous *Tshz1* transcriptional activation was examined via qPCR (Figure 24A,B). qPCR results showed that endogenous *Tshz1* mRNA transcript level was 30% higher than the mock control when RA-treated P19 cells were overexpressing E2F1 (Figure 24B). Having seen the enhanced *Tshz1* transcription by E2F1 overexpression, the effect of E2F1 depletion on *Tshz1*

expression was tested in parallel. Three siRNAs each targeting different sequence of endogenous *E2F1* were used to deplete endogenous E2F1 and their knockdown efficiencies were confirmed by immunoblot (Figure 24C). qPCR analysis was performed to see the changes in endogenous *Tshz1* transcript level. The result was consistent with overexpression result in that *Tshz1* transcript level was decreased by 30% compared to the *siCTL* group in RA-treated P19 cells.

Next, it was tested whether E2F1 also activates *Tshz1* promoter activity in RA-treated P19 cells by luciferase assays. Similar to qPCR result, *Tshz1* promoter activity was enhanced when cells were overexpressing E2F1 (Figure 25A,B). However, E2F1 overexpression had no effect on the promoter activity of *Tshz1* mutant that has mutation at a site where E2F1 is predicted to bind (Figure 25B). Overall, the enhanced transcriptional activation of *Tshz1* by E2F1 overexpression was observed with or without RA but the level of increase was much higher when cells were treated with RA.

Similarly, the effect of E2F1 depletion on *Tshz1* promoter activity was tested. Using P19 cells stably expressing luciferase constructs, E2F1 depletion resulted in significantly reduced promoter activity of $Tshz1^{-1500/+500}$ and $Tshz1^{-1250/-1200}$ but had no effect on luciferase activity of $Tshz1^{-1200/+500}$and $Tshz1^{Mut}$ (Figure 25C,D). These results indicate that E2F1

enhances *Tshz1* transcription possibly by activating its promoter activity.

Having confirmed E2F1's RA-dependent activation of *Tshz1*, I then sought to see if this effect was occurring in a cell division-dependent manner (Figure 26). To do this, I examined change in *Tshz1* promoter activity when cell divisions of E2F1-overexpressed (Figure 26A,B) or depleted (Figure 26C,D) cells were blocked by thymidine treatment. Luciferase assay results showed that *Tshz1* transcript level was reduced by half when cell division was blocked even if E2F1 was overexpressed. Also, E2F1 depletion did not further reduce *Tshz1* promoter activity when cell division was blocked by thymidine. These results imply that effect of E2F1 on *Tshz1* promoter activity requires cell division in RA-treated P19 cells.

To further validate the role of E2F1 as transcription factor of *Tshz1*, I tested to see if *Tshz1* promoter is occupied by E2F1. To see the direct binding of endogenous E2F1 to *Tshz1* promoter *in vivo*, chromatin immunoprecipitation assay (ChIP) using ChIP grade E2F1 antibody was performed (Figure 27). The result showed that E2F1 is chromatin-immunoprecipited with *Tshz1* promoter, which was strongly enhanced when cells were treated with RA. To further confirm, I used three different sets of primers, one targeting coding region of *Tshz1* (Figure 27A) and two targeting promoter regions of *Tshz1* (Figure 27B), when amplifying *Tshz1* gene from chromatin sample immunoprecipitated with E2F1 antibody. The results were

all consistent in that *Tshz1* gene was amplified in RA-dependent manner regardless of the primer targeting different regions of the promoter. To verify that the result was specific to *Tshz1* gene, I have also used the same ChIP samples to check the level of known targets of E2F1 (Figure 27C). The PCR result indicated that E2F1's known targets were also amplified in P19 cells but in RA-independent manner.

Next, ChIP assays were performed to see if endogenous E2F1 directly binds to *Tshz1* promoter in a RA and cell division-dependent manner. Cell division was blocked by thymidine treatment and it was tested whether E2F1 can still bind to *Tshz1* promoter (Figure 28A). The ChIP result using cell division-blocked P19 cells and E2F1 antibody showed the reduction of E2F1 binding to *Tshz1* promoter. Time-course ChIP assay also showed that E2F1 binds to Tshz1 promoter in a time-dependent manner, implying that E2F1 may require cell division in order to efficiently bind to *Tshz1* promoter in a RA-dependent manner (Figure 28B). Altogether, these results indicate that active cell division is required for E2F1 to activate *Tshz1* transcription in a RA-dependent manner. Overall, qPCR, luciferase, and ChIP assays suggest that E2F1 may act was transcription factor for *Tshz1*, triggering *Tshz1* expression during early neuronal differentiation in P19 cells (Figure 29).

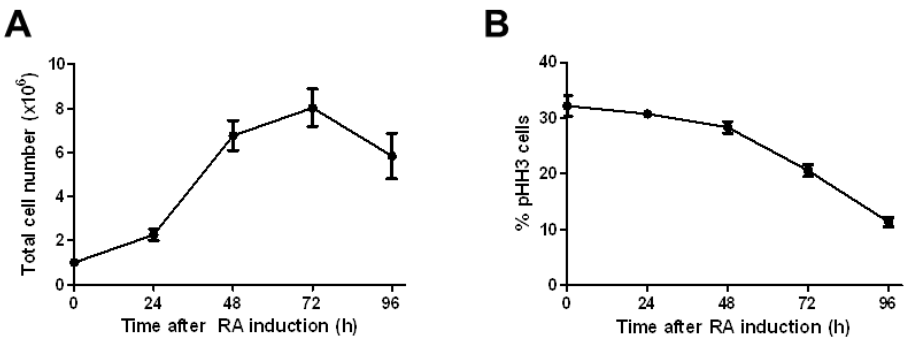


Figure 5. Time-course analysis of cell division in RA-treated P19 cells. P19 cells were treated with 1μM of RA and cultured up to 4 days.**(A)** The number of cells were counted at each day. **(B)** The cells were immunostained with the phospho-histone H3 antibody (pHH3). The number of pHH3-positive cells were counted. Data are shown as means ± S.D. (n = 3) and analyzed by One-way ANOVA with Tukey post hoc test. n.s. non-significant.

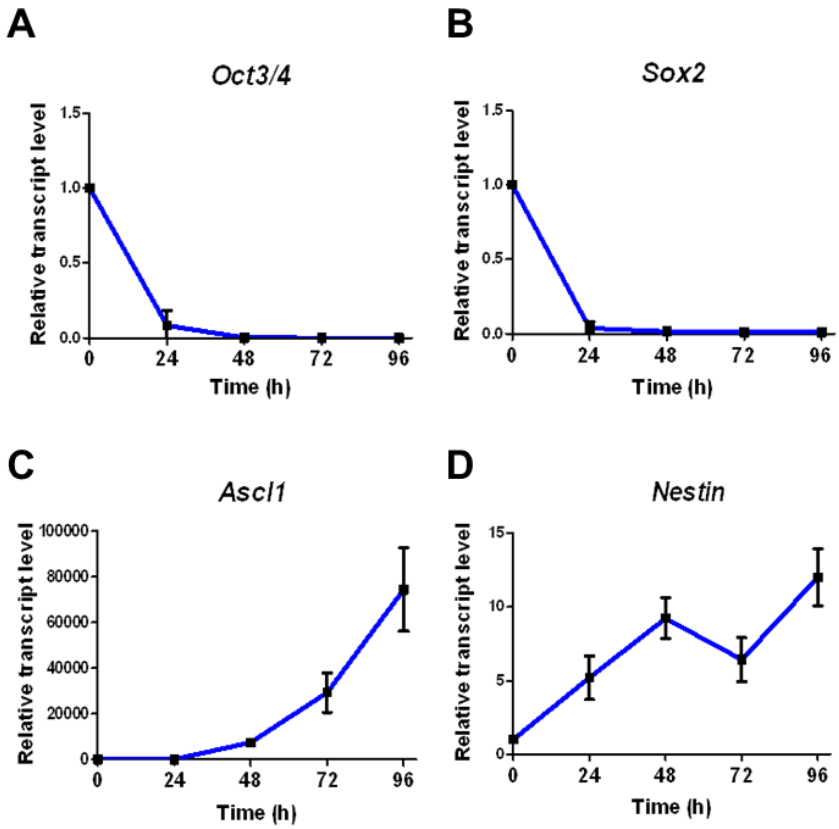


Figure 6. Expression of pluripotency and lineage-specific markers during neuronal differentiation in RA-treated P19 cells. (A-D) At indicated time points after RA treatment, the P19 cells were subjected to qPCR analysis with primers specific to selective pluripotency (*Oct3/4* and *Sox2*) and proneural markers (*Nestin* and *Ascl1*). The values are normalized with *GAPDH*.

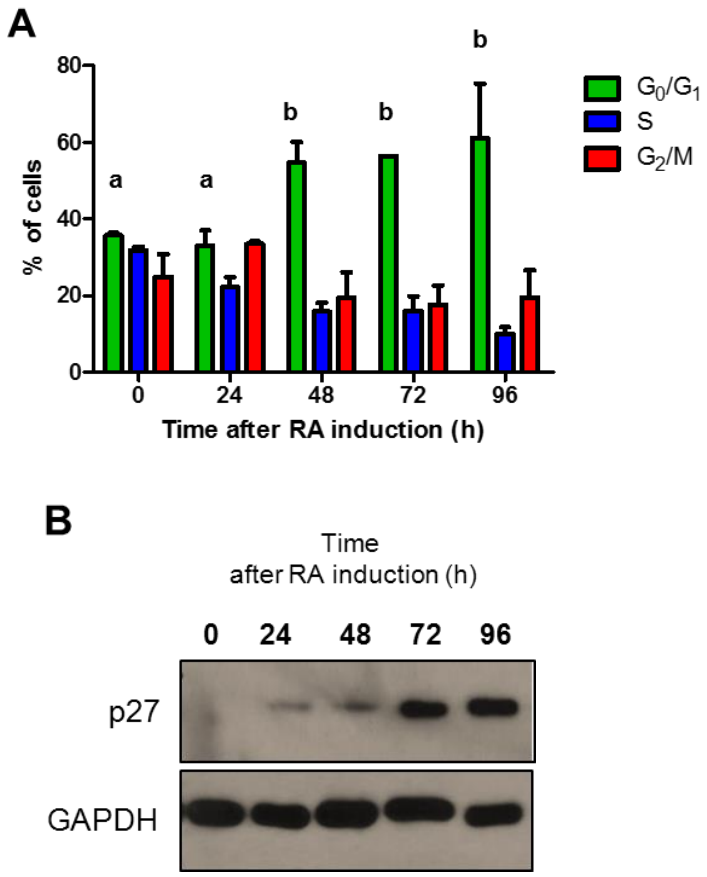


Figure 7. Time-course analysis of cell cycle profile in RA-treated P19 cells. (A) P19 cells were treated with RA and cultured up to 96 h. At indicated time points, the cells were subjected to FACS analysis to determine cell cycle stages. Data are shown as means ± S.D. (n = 3). Percentage of G_1 phase cells are analyzed by One-way ANOVA with Tukey post hoc test, and bars marked with different letters are statistically different from each other. **(B)** P19 cells were treated with RA and cultured up to 96 h. Immunoblot analysis of $p27^{Kip1}$each day. GAPDH was used as a loading control. Representative result of two independent replicates is shown.

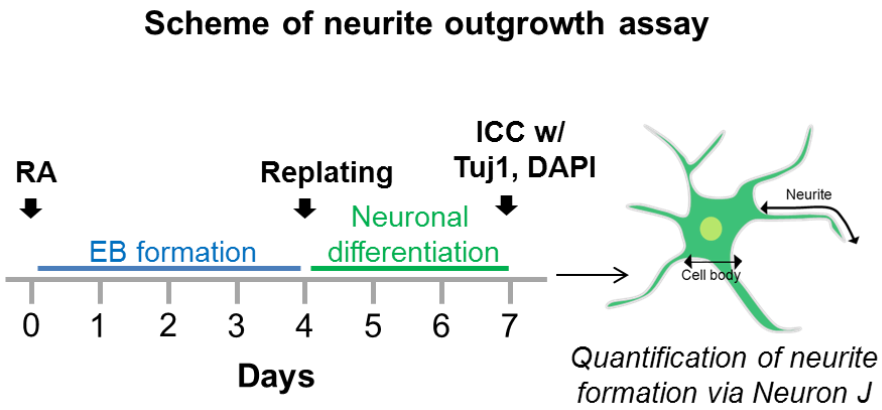


Figure 8. Experimental scheme of neurite outgrowth assay in P19 cells. Picture summarizes procedure for neurite outgrowth analysis performed in figure 5,6,7, and 12. RA-treated P19 cells undergo EB formation. Then, cells are dissociated and replated onto poly-L-lysine coated dish with B27 supplemented media to enforce the cells to become neurons for at least 3 days. Neurons are marked by immunostaining with Tuj1 antibody. Neurite length and branching are quantified using Neuron J program. Projection that is at least twice as long as the diameter of cell body is determined as neurite.

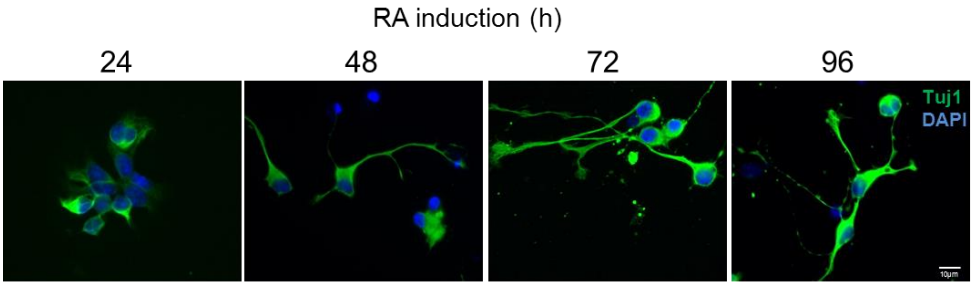


Figure 9. Time-course neurite formation and neuron maturation in RA-treated P19 cells. P19 cells were treated with RA and cultured up to 96 h. The cells were replated and enforced to form neurites for 3 days and immunostained with antibody against Tuj1 (green). DNA was stained with DAPI (blue). Representative images are shown. Scale bar, 10μm.

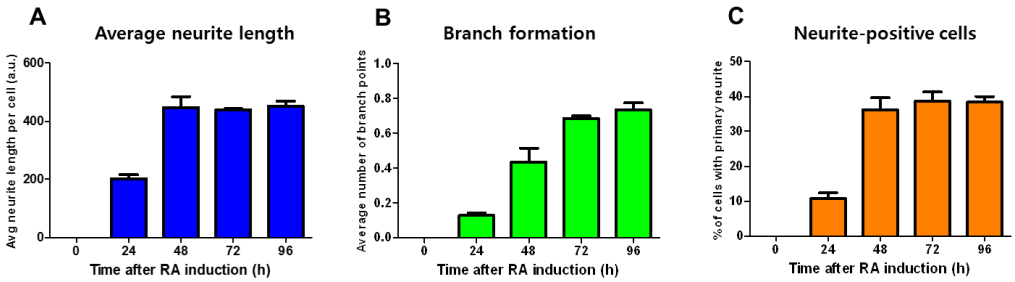


Figure 10. Time-course neurite outgrowth analysis of neurons in RA-treated P19 cells. P19 cells were treated with RA and cultured up to 96 h, followed by neuronal differentiation for 3 days. Neurites of Tuj1-positive neurons were analyzed by quantifying their number of cells with neurites (A), length (B), and branches (C) using Neuron J. Greater than 100 cells were quantified. **(A)** Tuj1-positive cells with extension formed from the cell body that is at least twice the cell body diameter was defined as primary neurite. **(B)** Primary neurite length was quantified using Neuron J, which was divided by total number of cells with neurites. **(C)** Branch formation was quantified by counting neurites that are extended from primary neurite. Average number of branches was calculated by dividing total number of branches by total number of cells with primary neurites. Data are shown as means ± S.D. (n = 3).

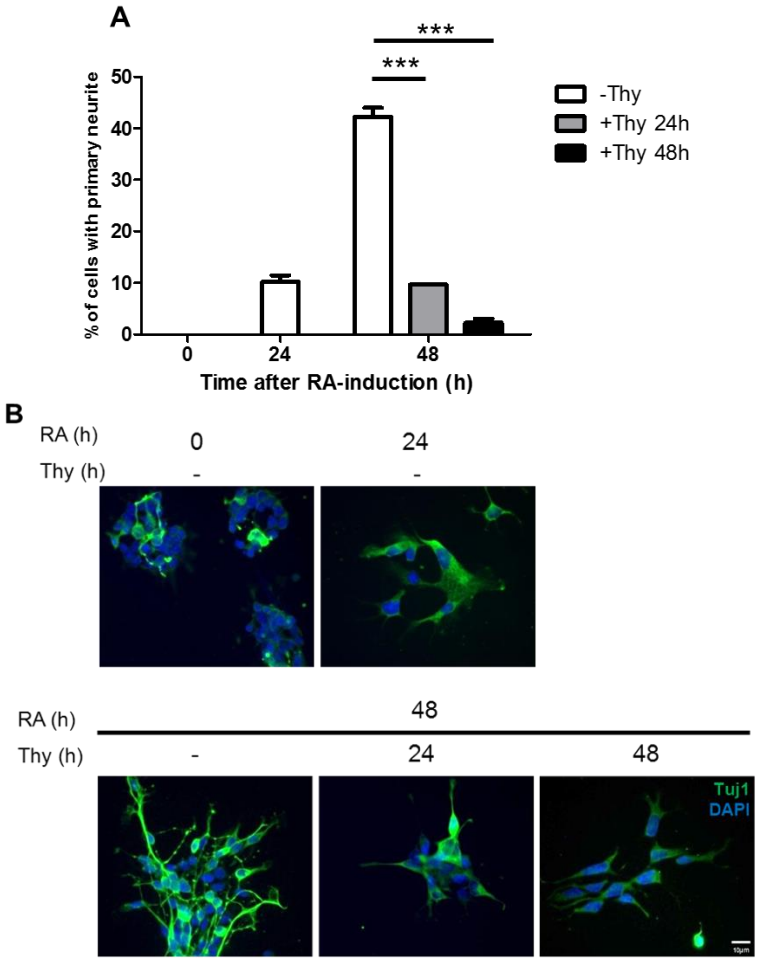


Figure 11. Effect of cell division block during early hours post RA induction on neurogenesis in RA-treated P19 cells. P19 cells were induced with RA in the presence of thymidine for 24 or 48 h. **(A)** The number of cells with primary neurites were counted and statistically analyzed. Greater than 100 cells were quantified. Data are shown as means ± S.D. (n = 3). *** p<0.001. Student t test. **(B)** Cells were immunostained with Tuj1 antibody and DAPI for DNA staining. Representative images of three independent replicates are shown. Scale bar, 10μm.

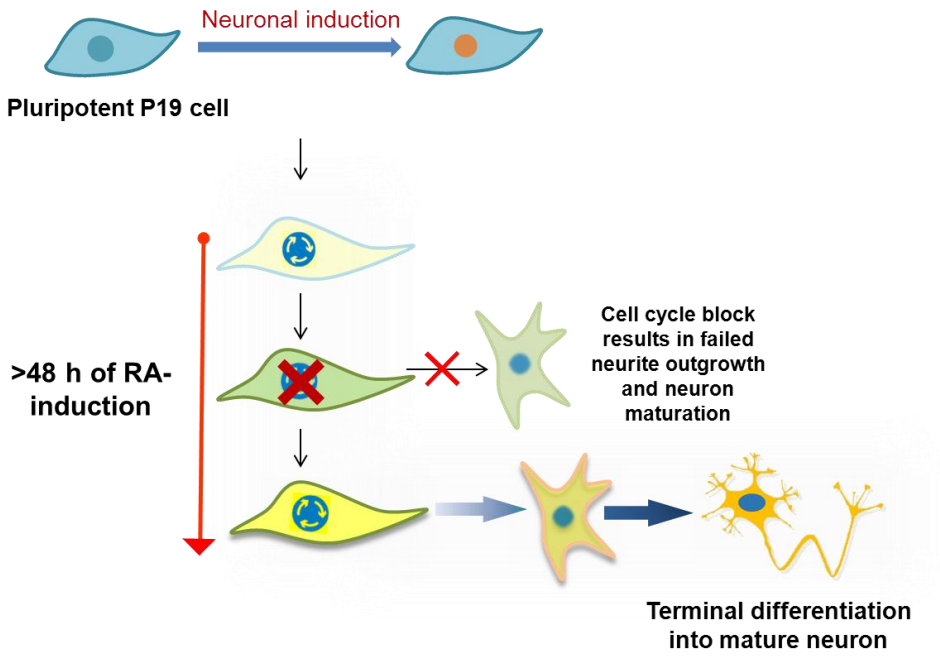


Figure 12. Summary of Part 1. The picture summarizes the results from Figure 1 to Figure 7. Analysis of cell cycle dynamics during neuronal differentiation suggests that at least 48 hours after RA treatment is needed for cells to differentiate into neurons. Cell division block using thymidine shows that cell division during early stage is important for neuronal differentiation in RA-treated P19 cells.

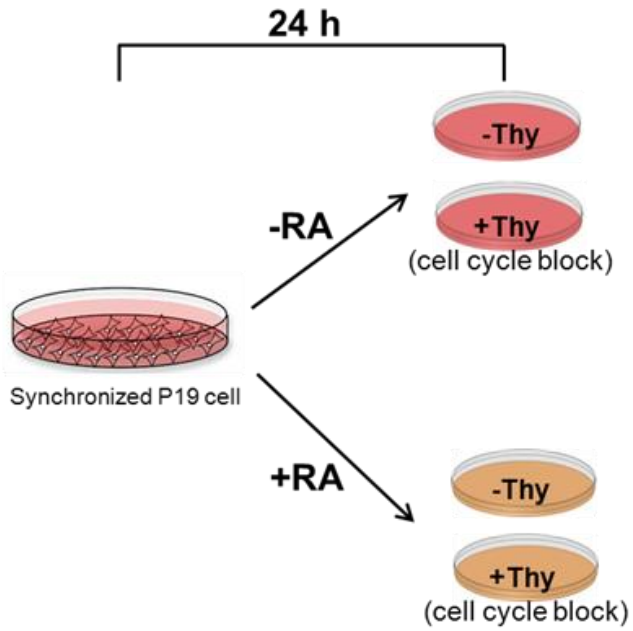


Figure 13. Experimental scheme of screening for cell division-dependent genes during early hours after RA treatment in P19 cells. P19 cells are synchronized by single thymidine block. Then, upon drug release, cells are grouped into four. In groups without RA induction, cells are either treated in the presence or absence of thymidine to see if the drug itself caused changes in the expression of genes. In groups treated with RA, cells are not treated with cell cycle blocking drug to see if the candidate gene is induced by RA. For cells treated with RA and thymidine, they are tested to see if their expression levels are affected by cell division block.

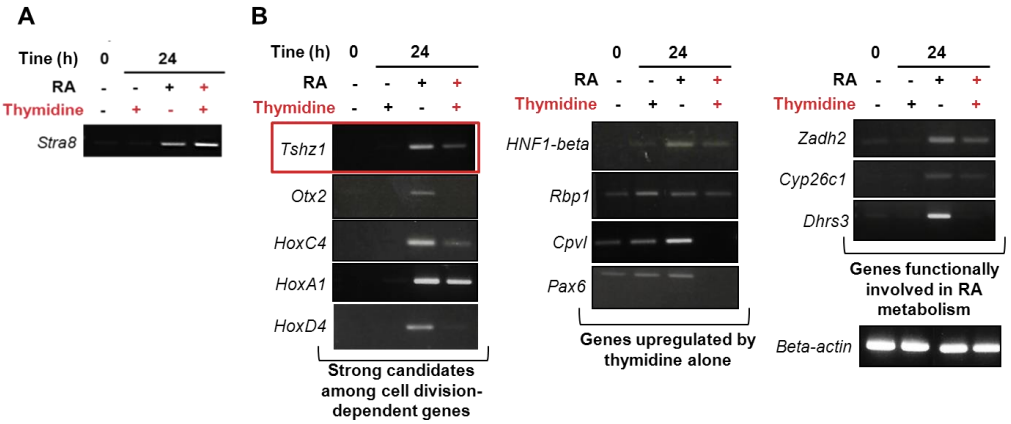


Figure 14. Summary of screening for candidate genes that are under cell division-dependent transcriptional control. (A) P19 cells were treated with RA for 24 h in the presence of thymidine, and subjected to RT-PCR analysis with primers specific to *Stra8*. **(B)** RT-PCR analyses were performed with primers specific to the indicated genes.

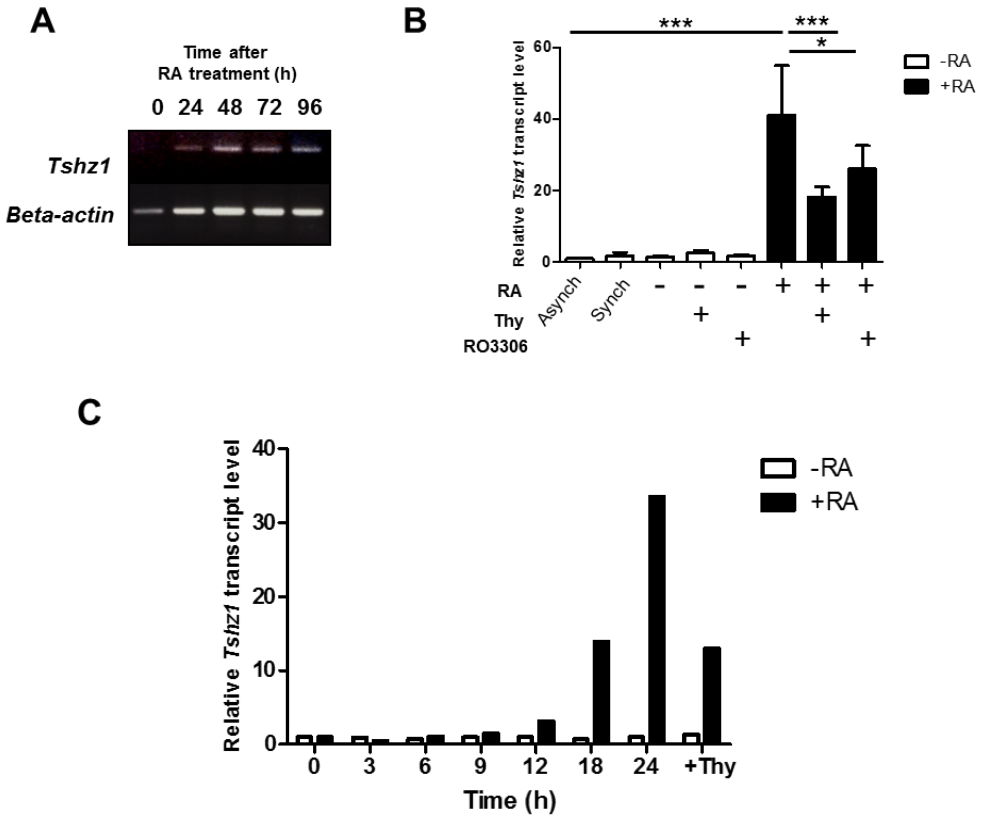


Figure 15. *Tshz1* is RA-induced gene that may be regulated in cell division-dependent manner. (A) P19 cells were treated with RA and RNA samples were harvested at indicated time points. RT-PCR analysis of *Tshz1* was performed. *Beta-actin* transcript level was used as a loading control. **(B)** P19 cells were treated with RA for 24 h in the presence of thymidine or RO3306, and subjected to qPCR analysis for *Tshz1* expression. Data are shown as means ± S.D. (n = 3). * p< 0.05, ** p<0.01, *** p<0.001. Student t test. **(C)** Synchronized P19 cells were RA-treated and cultured up to 24 h. Thymidine was also treated for 24 h. At indicated time points, the cells were harvested and subjected to qPCR analysis for *Tshz1*expression. The values are normalized with *GAPDH*.

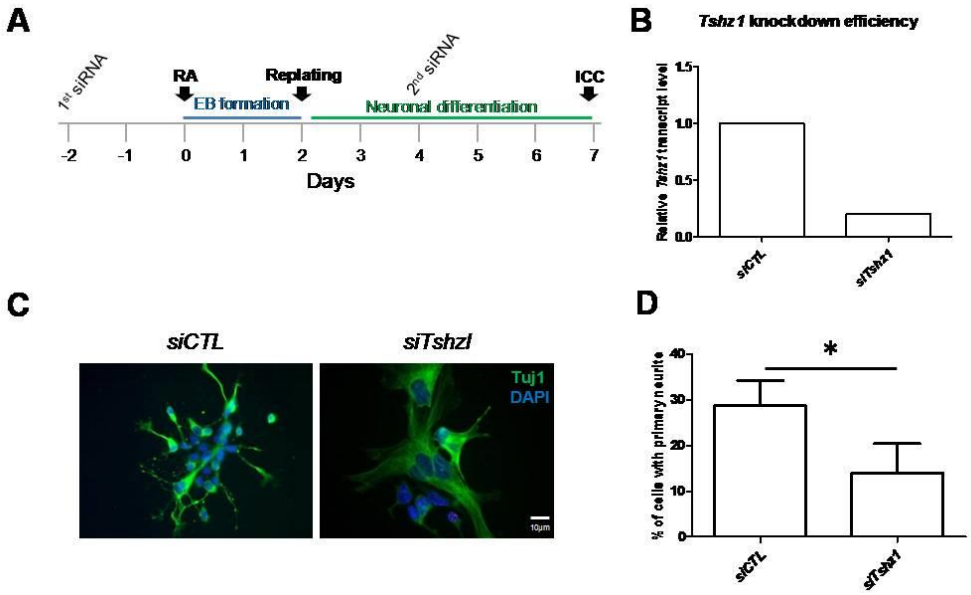


Figure 16. *Tshz1* depletion results in defect of neurite formation in RA-treated P19 cells. (A) Experimental scheme for neurite outgrowth assay with *Tshz1* depletion using siRNA. *siRNA* was treated twice, before and after neuronal differentiation. **(B)** P19 cells were transfected with *siTshz1* for 48 h and subjected to qPCR with primers specific to *Tshz1*. **(C)** The cells were immunostained with Tuj1 (green) antibody. DNA was stained with DAPI (blue). Scale bar, 10μm **(D)** Neurite outgrowth analysis of cells with *Tshz1* depletion. Percentage of neurite positive cells were quantified using Tuj1 immunostaining results and Neuron J. Data are shown as means ± S.D. (n = 3). * p< 0.05, Student t test.

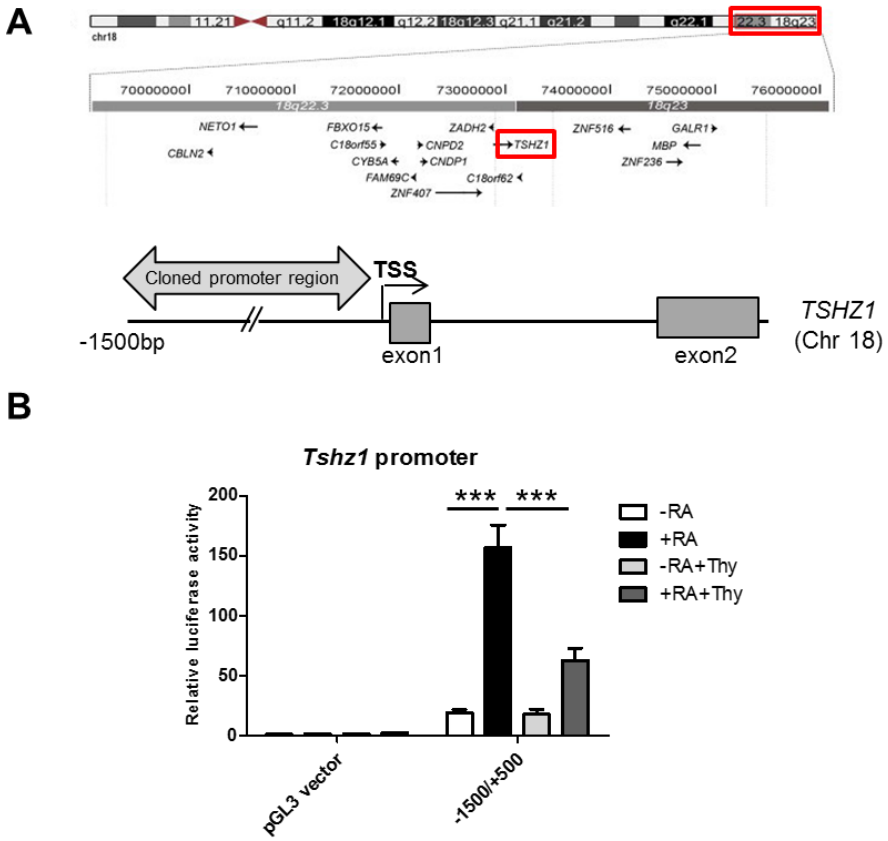


Figure 17. Promoter study of *Tshz1* in RA-treated P19 cells. (A) Diagram of genomic loci and cloned promoter region of *Tshz1*for the following promoter studies. -1500 bp relative to transcription start site (TSS) of *Tshz1* gene was cloned from P19 cells and subcloned into pGL3 luciferase vector. **(B)** Luciferase activity of *Tshz1* promoter in P19 cells. From-1500 bp to +500 bp region was linked to *luciferase* gene. P19 cells were stably transfected with the *Tshz1*-luciferase construct. Cells were treated with RA for 24 h in the presence of thymidine and subjected to luciferase assay. Luciferase activity of *Tshz1* promoter was measured relative to pGL3 vector that has basal luciferase activity. Data are shown as means ± S.D. (n = 3). *** p<0.001. n.s. non-significant. Student t test

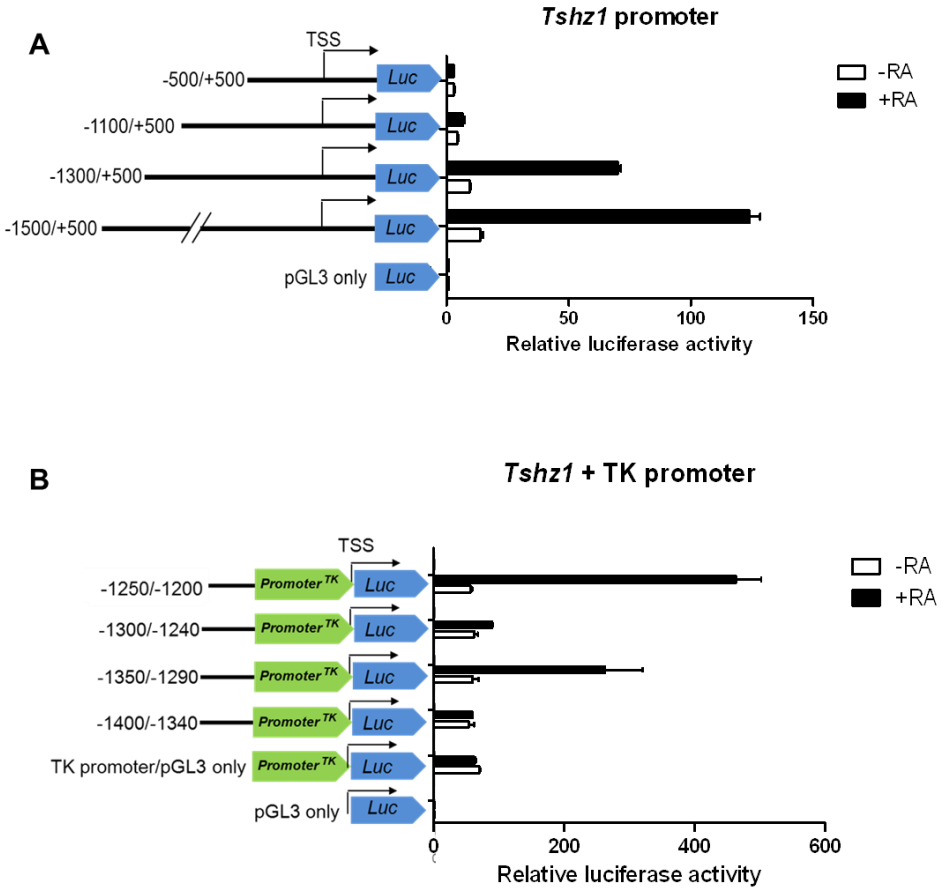


Figure 18. Identification of *Tshz1* promoter region that is essential for RA-induced transcriptional activation. (A) The *luciferase* gene was linked to the *Tshz1* promoter sequences up to -1500 bp. P19 cells were stably transfected with indicated *Tshz1*-luciferase constructs. **(B)** Fragments of the *Tshz1* promoter within -1400/-1200 region were linked to TK-luciferase constructs. P19 cells were stably transfected with the indicated *Tshz1*-TK-luciferase constructs. **(A-B)** Cells were treated with RA for 24 h and subjected to luciferase assay. Luciferase activity of *Tshz1* promoter was measured relative to pGL3 empty vector. Data are shown as means ± S.D. (*n* = 3).

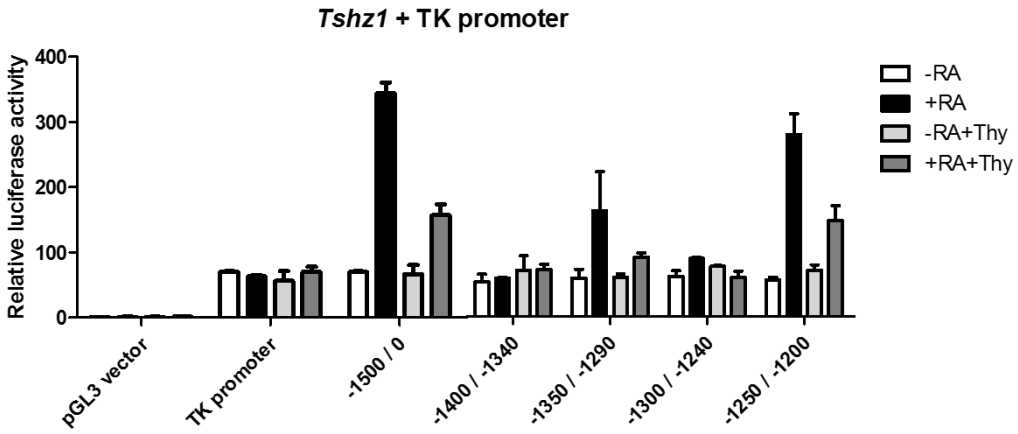


Figure 19. -1250/-1200 of *Tshz1* promoter region is essential for RA and cell division-dependent transcriptional activation. P19 cells were stably transfected with the indicated *Tshz1*-TK-luciferase constructs. Cells were treated with RA for 24 h in the presence of thymidine and subjected to luciferase assay. Luciferase activity of *Tshz1* promoter was measured relative to pGL3 empty vector. Data are shown as means ± S.D. (n = 3).

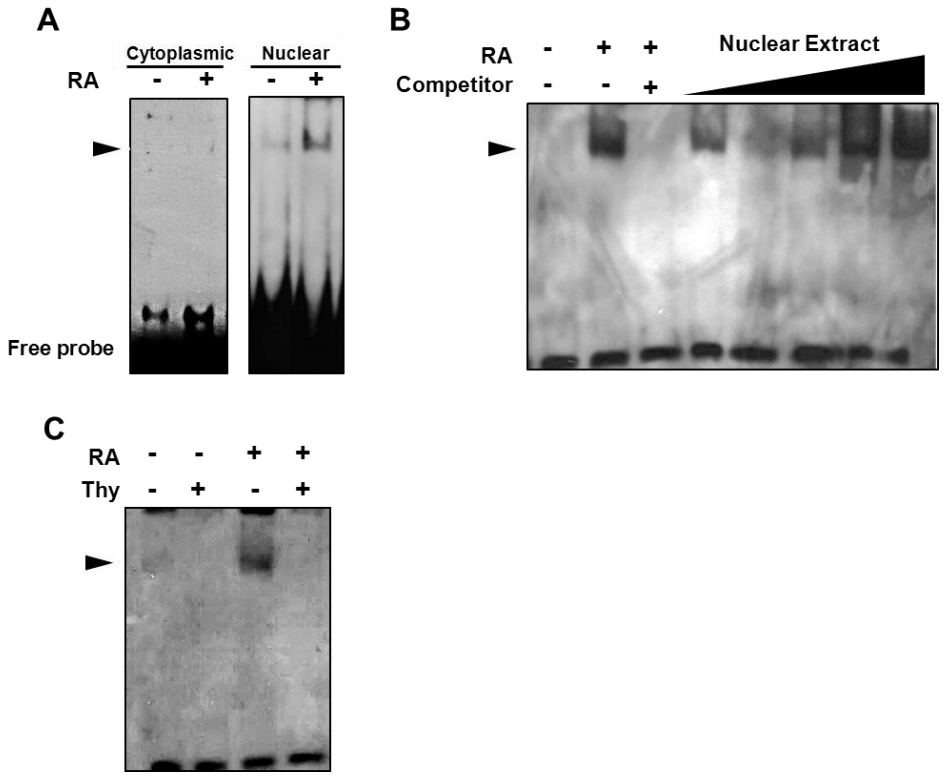


Figure 20. EMSA analysis with the *Tshz1* promoter$^{-1250/-1200}$ sequence. (A) EMSA assay was performed using -1250/-1200 of the *Tshz1* promoter as a probe. The cytoplasmic and nuclear extracts were prepared from P19 cells treated with RA for 24 h. Arrowhead indicates specific retarded band. **(B)** EMSA assay was performed with nuclear extract of P19 cells. As a competitor, excess amount (100 fold) of the cold probe was added into the reaction mixture. Increasing amount of nuclear extracts (4-24 μg protein) was added into the reaction mixture. **(C)** Nuclear extracts were prepared from RA-treated P19 cells which had been cultured in the presence of thymidine for 24 h.

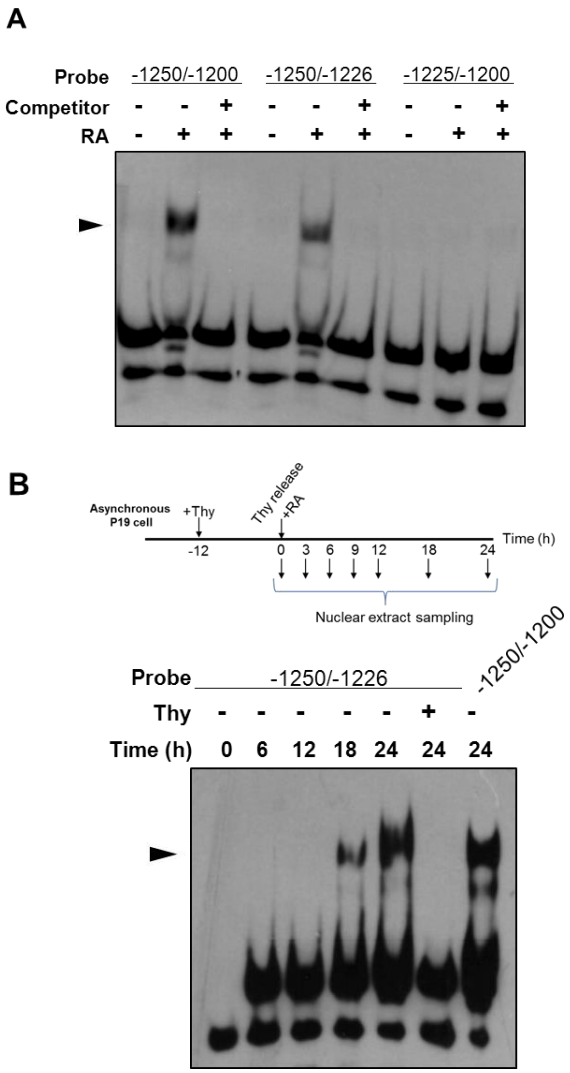
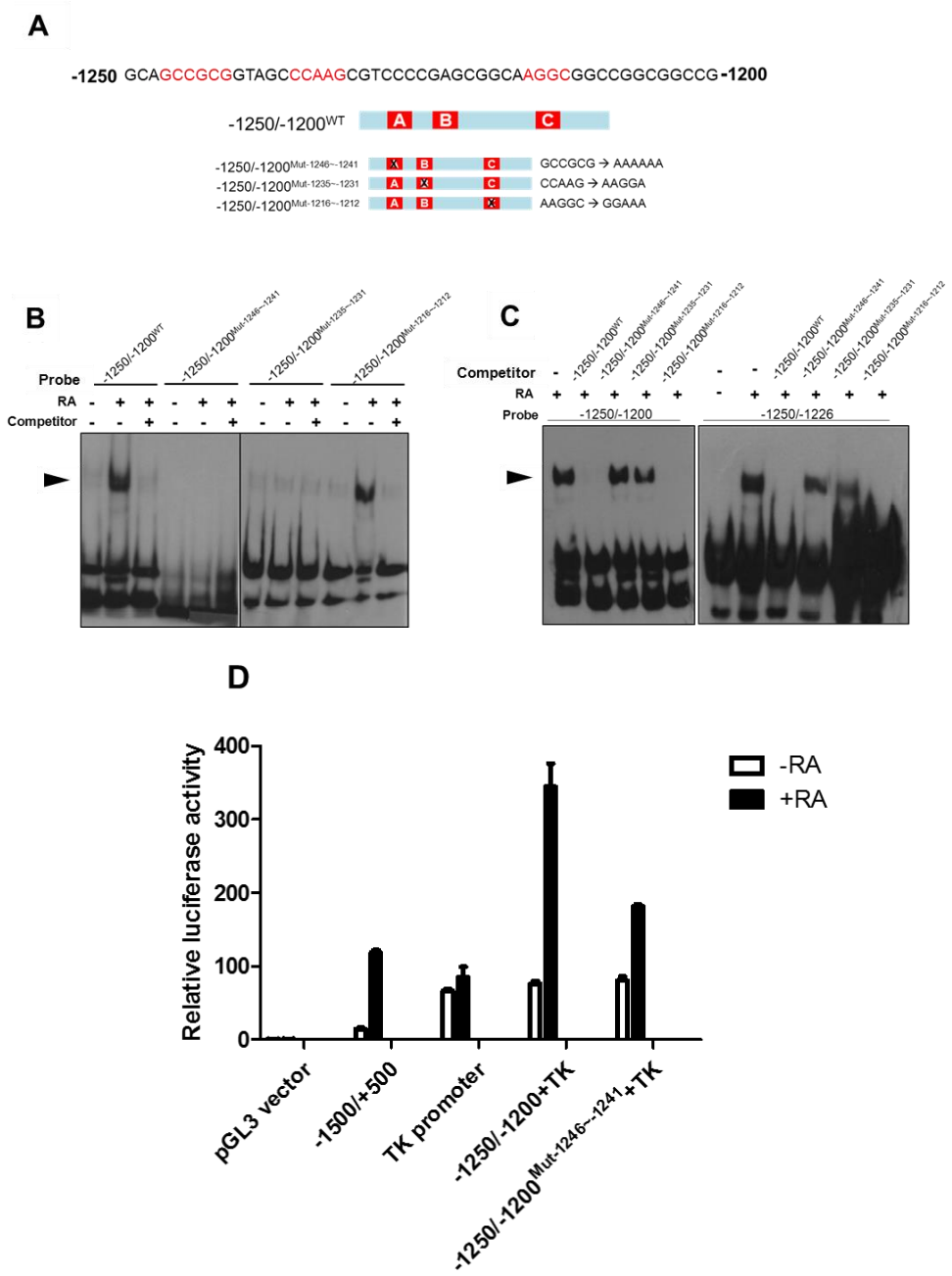


Figure 21. *Tshz1* promoter$^{-1250/-1225}$ is essential for RA and cell division-dependent binding of candidate nuclear factor. (A) EMSA assay was performed using three different sequences as a probe with nuclear extract of P19 cells that was RA-treated for 24 h. Competitor is a cold probe with same sequence as the indicated probe. **(B)** Time-course EMSA assay was performed using -1250/-1226 as a probe. Nuclear extracts of P19 cells treated with RA for indicated number of hours were each mixed with the probe. Arrowhead indicates specific retarded band.


A
-1250 GCAGCCGCGGTAGCCCAAGCGTCCCCGAGCGGCAAGGCGGCCGGCGGCCG -1200
-1250/-1200WT
-1250/-1200Mut-1246~-1241 GCCGCG → AAAAAA
-1250/-1200Mut-1235~-1231 CCAAG → AAGGA
-1250/-1200Mut-1216~-1212 AAGGC → GGAAA
B
Probe
RA
Competitor
C
Competitor
RA
Probe
-1250/-1200
-1250/-1226
D
Relative luciferase activity
400
300
200
100
0
-RA
+RA
pGL3 vector
-1500/+500
TK promoter
-1250/-1200+TK
-1250/-1200Mut-1246~-1241+TK

(continued)

Figure 22. Mutation analysis of *Tshz1* promoter$^{-1250/-1200}$ (A) Scheme showing potentially important sequences within -1250/-1200. Each region is labeled as either A, B, or C. Three different probes each containing mutation in either region are describe. The sequences of the mutants are indicated on the right side. **(B)** Wildtype with no mutation and three different mutants are used as probes. EMSA assay was performed using the indicated probes with nuclear extract of P19 cells that is RA-treated for 24 h. Arrowhead indicates specific retarded band. **(C)** Two probes, one with -1250/-1200 region and the other with -1250/-1226 region are used for EMSA assay. Probes are mixed with four different cold probes. Arrowhead indicates specific retarded band. **(D)** P19 cells were stably transfected with *Tshz1*-TK-luciferase construct containing mutation at -1246/-1241 region. Data are shown as means ± S.D. (n = 3).

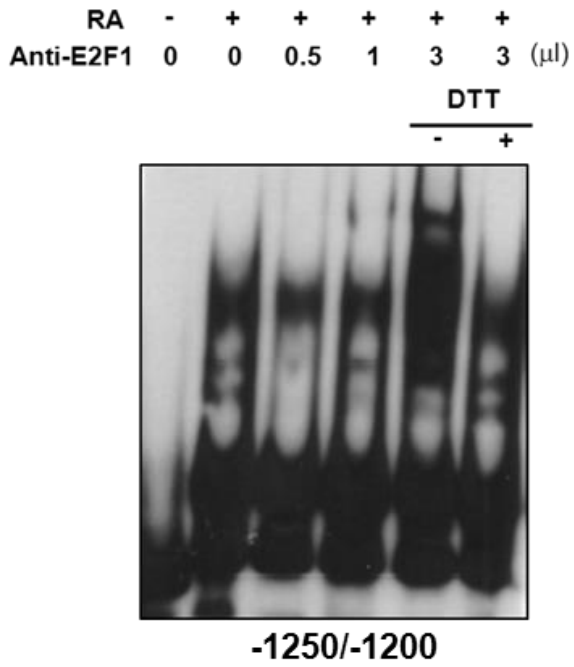


Figure 23. EMSA supershift analysis with the *Tshz1* promoter$^{-1250/-1200}$ sequence and E2F1 antibody. EMSA assay was performed with *Tshz1* promoter$^{-1250/-1200}$ as a probe. Nuclear extracts were prepared from the RA-treated P19 cells. Indicated amounts of the E2F1 antibody were added in the reaction mixture. DTT was also added to reduce the antibody interactions

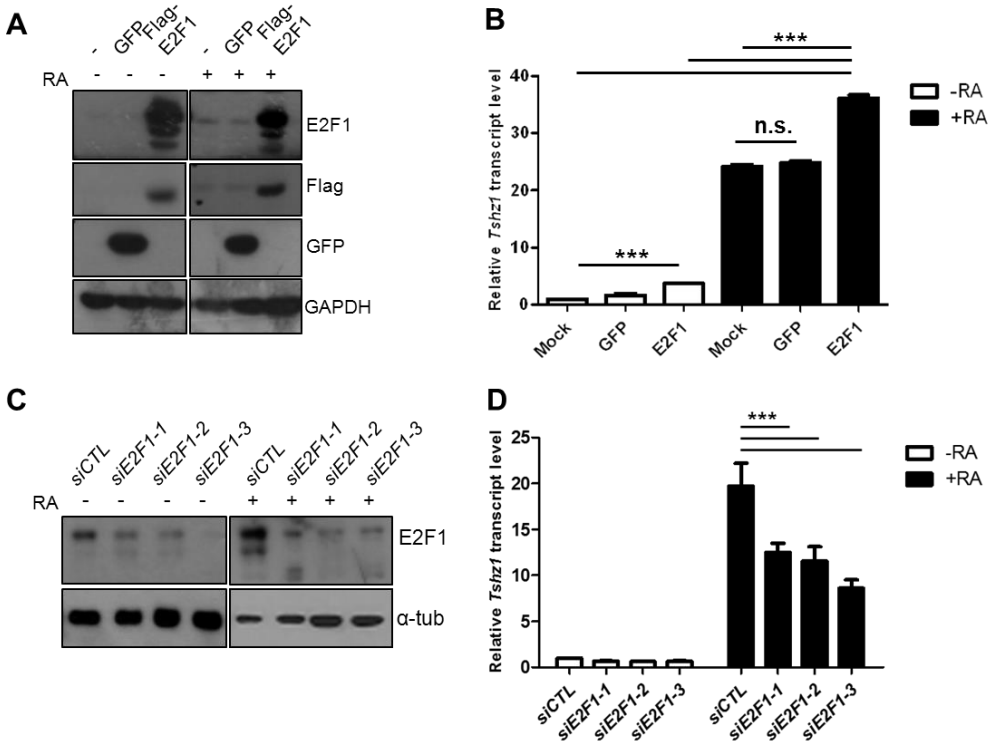


Figure 24. E2F1 enhances endogenous*Tshz1* transcription in RA-treated P19 cells. (A-B) P19 cells were transiently transfected with indicated plasmids for 24 h. **(A)** Protein level of transiently overexpressed flag-tagged E2F1. GAPDH protein level was used as loading control. **(B)** Transiently E2F1-overexpressing P19 cells were treated with RA for 24 h. Total RNA was extracted and subjected to qPCR to measure the endogenous *Tshz1* transcript level. mRNA level was normalized by the level of *GAPDH* and calculated as relative to –RA mock control group. **(C-D)** P19 cells were transfected with siRNA for 48 h. **(C)** Protein level of E2F1 was measured by immunoblot, and alpha-tubulin protein level was used as loading control. **(D)** E2F1-depleted P19 cells were treated with RA for 24 h. Total RNA was extracted and subjected to qPCR to measure the endogenous *Tshz1* transcript level. Data are shown as means ± S.D. (n = 3). Data are analyzed by One-way ANOVA with Tukey post hoc test, and bars marked with * are statistically different from each other. * p< 0.05, ** p<0.01, *** p<0.001. n.s. non-significant

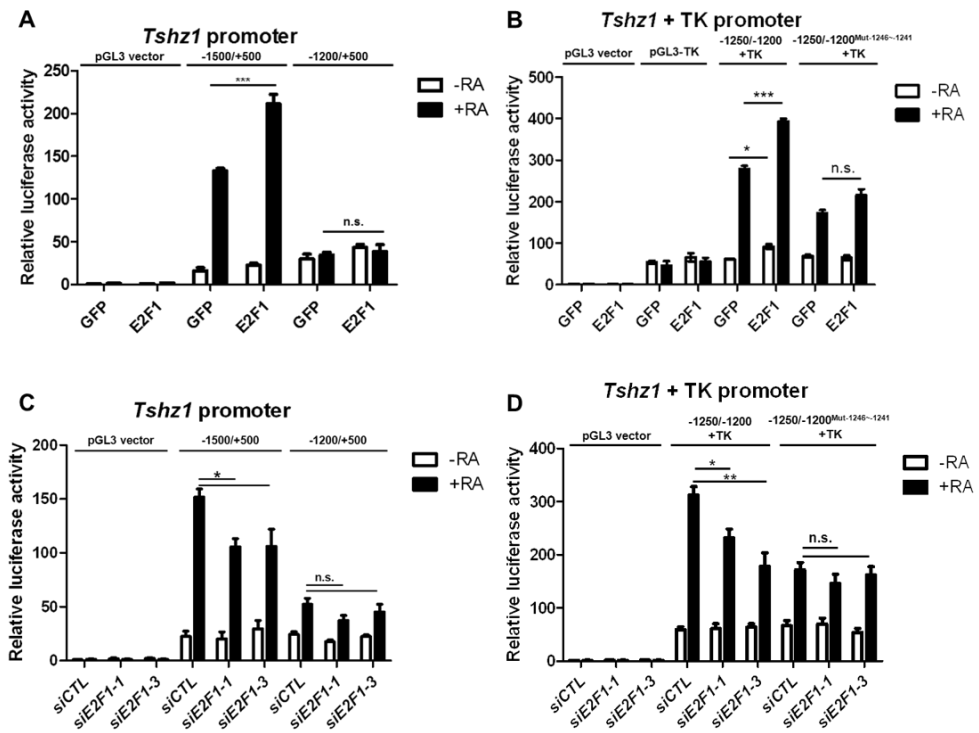


Figure 25. E2F1 promotes *Tshz1* promoter activity in RA-treated P19 cells. P19 cells were stably transfected with indicated *Tshz1* promoter constructs. **(A-B)** Transiently E2F1-overexpressing P19 cells were treated with RA for 24 h. **(C-D)** E2F1-depleted P19 cells were treated with RA for 24 h. Luciferase activity of (A,C) *Tshz1* promoter$^{-1500/+500}$and (B,D) *Tshz1* promoter$^{-1250/-1200}$ when RA-treated P19 cells had overexpressed or depleted-E2F1. Data are shown as means ± S.D. (n = 3). Data are analyzed by One-way ANOVA with Tukey post hoc test, and bars marked with * are statistically different from each other. * p< 0.05, ** p<0.01, *** p<0.001. n.s. non-significant

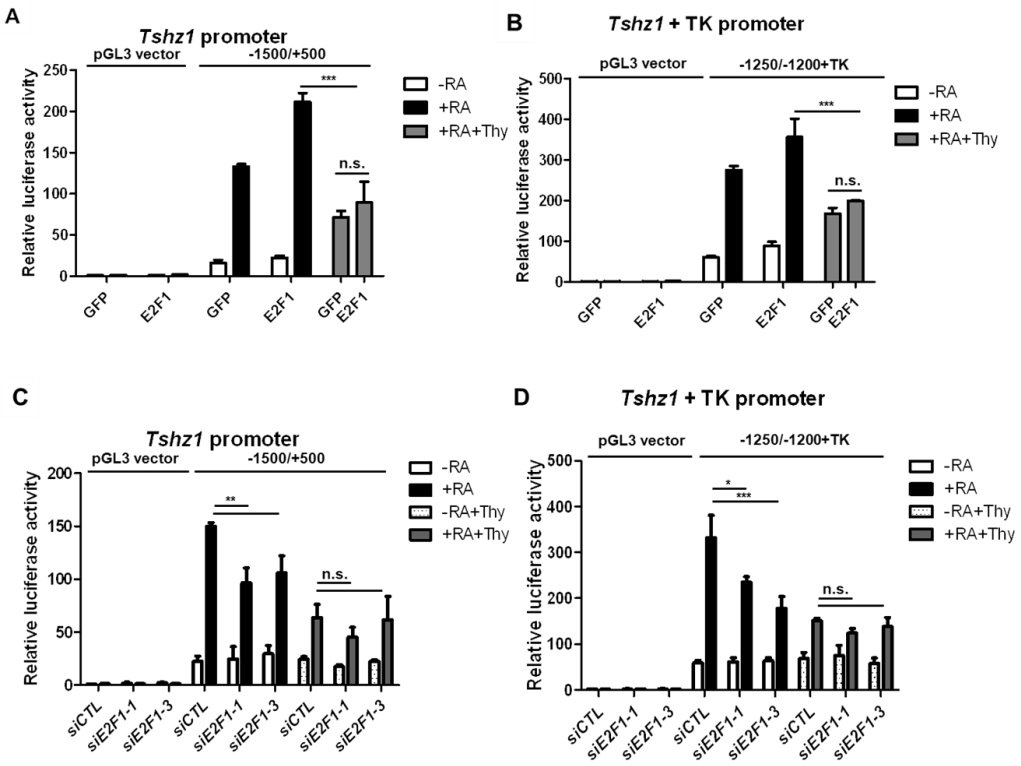


Figure 26. E2F1 may regulate *Tshz1* promoter activity in a cell division-dependent manner in RA-treated P19 cells. P19 cells were stably transfected with indicated *Tshz1* promoter constructs and treated with RA in the presence of thymidine for 24 h. **(A-D)** Luciferase assay result of (A,C) *Tshz1* promoter$^{-1500/+500}$ and (B,D) *Tshz1* promoter$^{-1250/-1200}$ in E2F1-overexpressed or E2F1-depleted cells. Data are analyzed by One-way ANOVA with Tukey post hoc test, and bars marked with * are statistically different from each other. * $p< 0.05$, ** $p<0.01$, *** $p<0.001$. n.s. non-significant

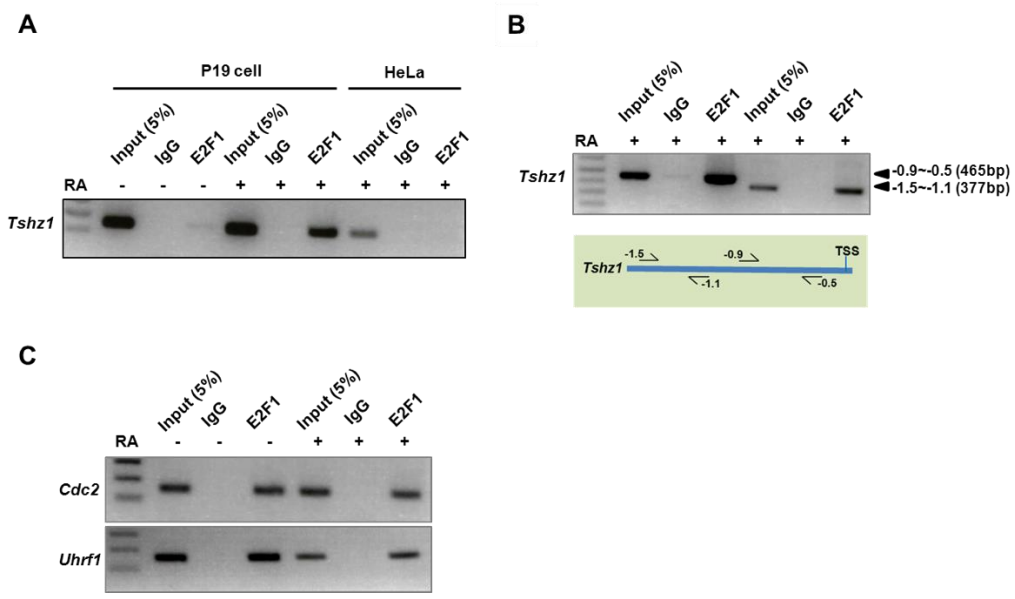


Figure 27. E2F1 directly binds to *Tshz1* promoter in RA-dependent manner in P19 cells *in vivo*. (A-C) Nuclear extracts were prepared from P19 cells which had been treated with RA for 24 h. **(A)** ChIP-PCR analysis was performed with E2F1 antibody and PCR using primers specific to a coding sequence of *Tshz1*. **(B)** ChIP-PCR analysis was performed with primers for -1.5/-1.1 and -0.9/-0.5 of the *Tshz1* promoter. **(C)** ChIP-PCR analysis of E2F1 were performed with primers specific to *Cdc2* and *Uhrf1* genes.

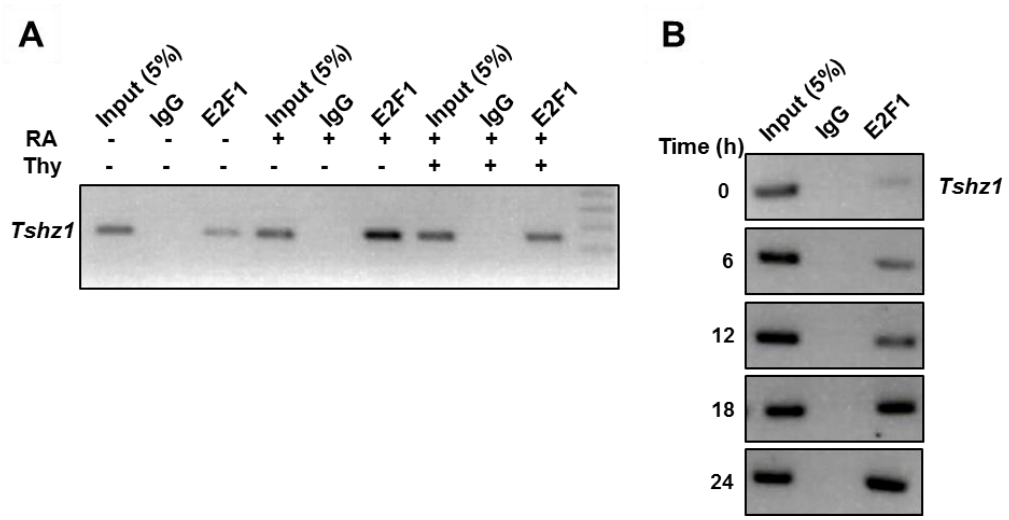


Figure 28. E2F1 directly binds to *Tshz1* promoter in cell division-dependent manner in RA-treated P19 cells *in vivo*. (A-B) Nuclear extracts were prepared from P19 cells which had been treated with RA up 24 h. **(A)** P19 cells were treated with RA in the presence of thymidine for 24 h. ChIP-PCR analysis was performed with E2F1 antibody and PCR using primers specific to -1.5/-1.1 region of *Tshz1* promoter. **(B)** Nuclear extract was prepared at indicated time points after RA treatment. ChIP-PCR analysis was performed with E2F1 antibody and target regions were amplified using primers specific to -1.5/-1.1 region of *Tshz1* promoter.

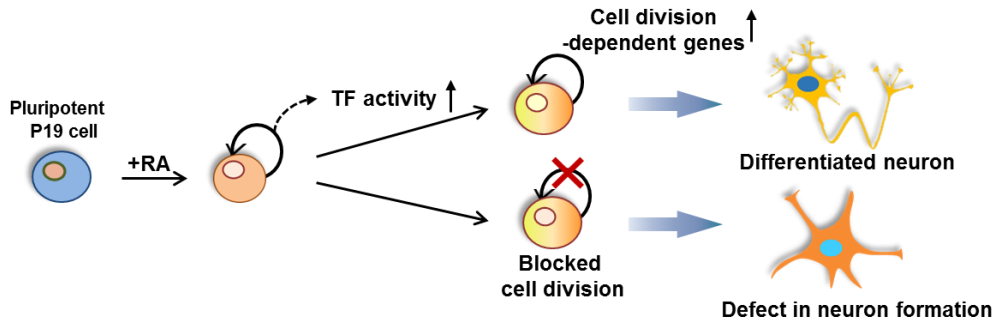


Figure 29. Model. Cell divisions after RA-induced neuronal differentiation in P19 cells are required for formation of neurons. E2F1 is one of transcription factors that may act in cell division-dependent manner to activate its downstream target gene, *Tshz1*, which is also important for neuronal differentiation.

Discussion

Discussion

In this study, the main goal was to find direct evidence on the link between cell division and differentiation. Using RA-induced neuronal differentiation system of P19 cells, I propose that cell division during early hours of RA induction is necessary for expression of genes that are important for neurogenesis. By screening for RA-induced genes that cannot be induced without cell division during the early hours, I was able to discover several genes that are regulated in a cell division-dependent manner. One of the genes, *Tshz1*, a product of which is zinc finger DNA-binding transcription factor that is known to be important for early development in several species, was selected for in-depth study. Promoter analysis revealed a specific region that is required for RA-dependent transcription. Sequence mutation and computational prediction analysis listed potential upstream transcription factors for *Tshz1*. Finally, I show that E2F1, a transcription factor important for G1/S transition, positively regulates *Tshz1* transcription in a RA and cell division-dependent manner by physically binding to its promoter.

Thymidine and RO3306, drugs that arrest cell cycle at S and G2 phase, respectively, by interfering with different pathways, are used to block cell division throughout the study. The main technical shortfall of the method is using pharmacological means to control cell division. One of the reasons

for this is that cell cycle and cell division are intricately regulated process that are connected to other major cellular process, such as cell growth, DNA replication, and cell death. Possibility that drug treatments could have not only blocked cell division, but also affected other pathways should always be taken into consideration when making conclusions from these data. Nevertheless, I have used the same drug treatment condition for various assays in the study and produced consistent results. Also, I frequently supported data from experiments using these drugs with time-course assays to confirm the cell division-dependency. The study needs more natural setting to examine the pathway controlled by cell division, which could be done using FUCCI cell line in the future.

In this study, I used total cell counting, FACS, and well-known cell cycle-related markers to examine cell division-related changes during RA-induced neuronal differentiation. These data collectively suggest that cells are actively undergoing cell division until 48 hours after RA induction, after which cell division dramatically decreases and cell cycle arrest increases on a population level. Time-course assay of pluripotency and lineage specific markers showed that differentiation status change with similar pattern. These overall patterns of cell cycle dynamics and differentiation suggested that subcellular changes during early stage might be important for neuronal differentiation. In order to test this projection, direct correlation between the

events within the same cell should be examined in detail, nevertheless, similar time-course assays were reported using hESCs *in vitro* differentiation system. It was observed that earliest implementation of differentiation into NE (neuroectoderm) involves restructuring of the cell cycle (Jang et al., 2016). If possible, it would be interesting to see what portions of cells are undergoing these changes and which cell division-related events determine these variations despite the same environment during differentiation.

Due to limitations on obtaining good quality Tshz1 antibody, the study could not fully explain how Tshz1 might play role during neuronal differentiation in P19 cells. Tshz1's potential role during differentiation can be inferred from its function in other tissue development. *Tshz1* was suggested as downstream target of Pdx1 during beta pancreatic cell differentiation (Raum et al., 2015). Through analysis of *Tshz1* knockout mice, the group suggested that Tshz1 may regulate beta-cell maturation but not endocrine progenitor expansion. In CNS, a group found that Tshz1 was necessary for migration of neuroblasts in the OB as well as for differentiation of granule cells and a subclass of periglomerular interneurons (Ragakocova et al., 2016). These studies imply that Tshz1 might have innate character to promote undifferentiated cells and acquire its cell fate, possibly by being involved in controlling its downstream transcription network.

There is a high possibility that E2F1 is not a sole upstream activator

of *Tshz1*. E2F1 knockdown does not completely abolish endogenous *Tshz1* transcription and other predicted candidate transcription factors still need to be tested in the future. Also, there has been a study showing that *Tshz1* is induced by RA in mouse embryo and that it can be chromatin-immunoprecipitated by RAR-gamma (Savory et al., 2014) but the binding region was about -9Kb upstream relative to TSS. This suggests that there are other upstream regulators for *Tshz1* transcription in RA-dependent manner during development.

E2F1 is a well-known cell cycle-regulating transcription factor, the activity of which is regulated by retinoblastoma protein family during G1 phase. In addition to its role during conventional cell cycle, the involvement of E2F1 during tissue development and stem cell differentiation has been suggested by several previous studies. Studies on *E2F1* deficient mice brain showed decreased stem cell and progenitor division in the proliferative zones of the lateral ventricle wall and the hippocampus in postnatal brain (Christiana et al., 2002). Reduction in neuronal cell numbers due to lack of E2F1 was observed in granule cells in the olfactory bulb, dentate gyrus, and cerebellum. During early germ layer specification in hESCs, E2F1 was found to form complex with cyclin D1 on endoderm promoters (Pauklin et al., 2015). The functional significance was that E2F1 may be involved in transcriptionally repressive interaction with cyclin D1 in late G1 phase. In

the case of early pancreas development, Cdk4 was found to be important for expansion of Pdx1+ pancreatic progenitor cells. E2F1 was implied as Cdk4-mediator, which promotes beta cell progenitor expansion by transcriptionally activating Ngn3 promoter (Kim and Rane, 2011). Also, several E2F family members were examined during adipogenesis using 3T3-L1 cell line. The study revealed that E2F4 plays repressive role, whereas E2F1 exerts activation of PPAR-gamma expression upon the initiation of mitotic clonal expansion (Fajas et al., 2002). Similar to the implications of my study, discovery of transcriptional role played by E2F1 during adipogenesis was represented as the mechanistic link between proliferative signaling pathway and steps involved in adipocyte differentiation.

Despite these previous studies that support the potential role of E2F1 during early stages of neuronal differentiation in RA-induced P19 cells, additional experiments are required to fully understand the involvement of E2F1. For example, E2F1 activation is known to require heterodimerization with DP (dimerization protein) family members (Poppy Roworth et al.,2014). Depending on the type of heterodimerization that is formed, it forms larger complex containing retinoblastoma family members, which directs E2F1 either to activate or repress its target genes (Dimova and Dyson, 2005). Therefore, it is important to examine with which coregulators E2F1 form heterodimer and whether or not other members of E2F family is involved in

Tshz1 transcription.

Important question that arises from the study is how E2F1 regulates *Tshz1* transcription in cell division-dependent manner. ChIP assay and EMSA supershift assay both suggest that E2F1 binds to Tshz1 promoter region in a sequence-specific manner but this does not explain how E2F1 recognizes Tshz1 to be its downstream target by coordinating with cell division. E2F1 is well characterized protein that is important for cell proliferation even in non-differentiating cells. It is known that all members of E2F family proteins require dimerization partners either to activate or repress their target genes (Fajas et al., 2002; Poppy Roworth et al., 2017). E2F1 may switch its sets of target genes by interacting with different partners depending on upstream cues. It has been investigated that E2F1 recruits HAT (histone acetyl transferase) to local chromatin, which results in gene expression control (Kheir and Lund, 2010). In this way, E2F1 may function during differentiation but in a context specific and cell type specific manner, possibly by dimerizing with different factors (Dimova and Dyson, 2005).

What is the biological significance of linking differentiation to cell cycle progression? Cell fate is achieved by expressing specific genes that are required for acquiring a specific identity of the cell. One of the direct ways which control these gene expressions is by altering epigenetic status that

globally regulates which genes to be turned on or off. Differentiation, a process by which stem cells acquire its final cell fate, accompanies this high level of gene expression changes via chromatin remodeling. During normal cell cycle, epigenetic marks are transiently erased during S phase to duplicate genes as well as histones that comprise chromatin to produce identical daughter cell with same epigenetic state. In this way, progression through the cell cycle during differentiation allows the cell to undergo chromatin remodeling to regulate gene expression. Because changes in global transcriptome during differentiation require chromatin restructuring, differentiation is tightly linked to cell cycle phase, which allows for re-establishment of histones (Ma et al, 2015).

The link between cell cycle-dependent epigenetic changes during differentiation has been recently investigated by several reports. A group has shown that G1 phase-expressed genes are heavily enriched for roles in development and cell-fate commitment. This change in gene expression was found to be linked to the cytosine modification 5-hydroxymethylcytosine (5hmC), which was increased during late G1 and re-established during G2 phase (Singh et al., 2013). This and other recent studies imply that there might be a cell cycle-regulated active chromatin restructuring during S phase of stem cell differentiation. Although this idea has not been tested in depth, there might be a layer of system that controls chromatin remodeling through

cell division, thereby efficiently controlling homogenous gene expression and differentiation of generations of cells in a lineage-specific manner.

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국문 초록

세포주기의 진행은 미분화된 줄기 세포의 운명을 결정짓는 과정과 맞닿아 있다. 따라서 세포 분열은 줄기세포의 분화가 정상적으로 진행되는 데에 필요한 주요인자 중 하나로 볼 수 있는데, 예를 들어 지방세포 분화 과정 동안 mitotic clonal expansion (MCE)에 대한 연구를 통해 그 중요성이 시사 된 바 있다. 하지만 세포분열이 조직발생 및 항상성 유지를 위하여 다양한 분화과정에서 역할을 하는지 여부에 대해서는 심도있는 이해가 부족하다. 이와 같이 세포분열과 분화에 대한 연관 관계가 있음에도 불구하고 분화과정에서 세포분열의 직접적인 역할과 작용기전에 대해서는 알려진 바가 없다. 본 연구에서는 신경세포로의 분화과정 동안 세포분열의 역할에 대하여 연구하였다. 이를 위해 레티놀산 (retinoic acid) 로 신경세포분화를 유도한 P19 세포주의 *in vitro* 분화 시스템을 이용하여 세포분열과 신경세포분화과정의 직접적인 관계여부를 확인하고자 하였다. 이를 위해 세포분열을 저해하는 약을 처리하였을 때 P19 세포가 신경세포로 분화하지 못하는 것을 관찰하였다. 본 현상에 대한 기전을 연구하기 위해 세포분열 저해제를 처리하였을 때 발현이 저해되는 유전자를 스크리닝 하여 *Tshz1* 이

세포분열 의존적으로 발현됨을 확인 할 수 있었다. *Tshz1* 의 촉진유전자를 클로닝하여 발현에 필수적인 부위를 분석한 결과, *Tshz1* 의 특정 부위가 P19 세포에서 레티놀산 그리고 세포 분열 의존적으로 발현되는 데에 중요함을 알 수 있었다. 컴퓨터 프로그래밍을 이용해 동정한 특정 부위의 서열을 분석하여 *Tshz1* 의 전사인자로 작용 가능한 후보군을 설정하였는데, 이전 보고된 논문들을 기반하여 E2F1 이 *Tshz1* 의 전사인자로 작용할 수 있는 가능성을 확인하였다. 이것을 실험해보기 위해 E2F1 의 발현을 억제하거나 과발현 한 후 *Tshz1* 의 발현 정도를 다양한 방법을 통해 확인한 결과, E2F1 이 *Tshz1* 의 전사인자로 작용할 수 있음을 알 수 있었다. 또한 약 처리를 통해 세포분열을 저해하였을 때 E2F1 이 *Tshz1* 의 유전자에 결합하여 전사인자로 작용하는 효과가 감소하였다. 종합적으로 신경세포분화과정 동안 세포 분열은 E2F1 전사인자로의 작용을 통하여 하위 유전자인 *Tshz1* 의 발현을 조절하고 이러한 일련의 과정을 통해 신경세포분화에 중요한 역할을 함을 알 수 있었다. 본 연구는 세포 분열 의존적으로 조절되는 유전자의 동정과 상위 기전을 규명함으로써 세포분열이 신경세포분화 과정을 조절할 수 있음을 시사한다.

주요어: 줄기세포, 분화, 세포 분열, 레티놀산, 전사인자, 신경세폽분화, P19 배아줄기암세포

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